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THE
BIOLOGICAL BULLETIN

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MARINE BIOLOGICAL LABORATORY WOODS HOLE MASS.

THE OCCURRENCE OF A DIABETOGENIC FACTOR IN THE EYESTALKS OF CRUSTACEANS*

A. A. ABRAMOWITZ, F. L. HISAW AND D. N. PAPANDREA

(*Woods Hole Oceanographic Institution, Woods Hole, Massachusetts*)

During an investigation of an endocrine influence on carbohydrate metabolism of crustaceans, it was found that aqueous extracts of the eyestalks produced within a very short time an intense hyperglycemia in the blue crab, *Callinectes sapidus*. This observation had been made several weeks previously by Mr. J. Armstrong [1] in the crayfish, and it now appears that a diabetogenic factor may exist in the eyestalks of the decapod crustaceans at least, and that it may be an agent in the normal regulation of sugar metabolism in this group of animals.

Of the common large decapods of the Woods Hole area the lobster has been found to be a very suitable form for such an investigation, but owing to legal difficulties in obtaining this species the blue crab was used. Blood was obtained by heart punctures made through a small hole drilled in the carapace, and subsequently analyzed by the method of Miller and Van Slyke.[2] The use of an anticoagulant was found unnecessary. The blood sugar values represent the total reducing substances, and for the present no distinction between fermentable and nonfermentable sugar was made. The amount of blood withdrawn for each analysis was 0.5 cc.

The blood sugar values of crabs freshly brought into the laboratory vary considerably. As an example, the individual values of seven crabs were 16, 112, 52, 18, 60, 27 and 55 mgs. per cent. This considerable variation is due to the handling of the animals, for it disappears when the crabs are segregated into separate containers and left undisturbed. After 5 days of isolation and starvation, the blood sugar values fell gradually, so that on the fifth day the individual readings of the five crabs showing the highest of the above values were 25, 24, 22, 24 and 21 mgs. per cent. Excess handling of the animals or other forms of excitement tends to induce hyperglycemia even after segregation and fasting. Consequently, blood samples were obtained as rapidly as possible and with a minimum of disturbance to the animals. When extracts were to be tested for glycemic effects, the animals

* Contributions from the Woods Hole Oceanographic Institution, No. 329.

[1] Personal communication.

[2] Miller, B. F. and D. D. Van Slyke, 1936. *Jour. Biol. Chem.*, 114: 583.

were always separated into individual containers and starved for at least 5 days previously.

1. The Effect of a Single Injection of Eyestalk Extract

Twenty-one crabs were prepared as previously described. The average blood sugar value for this group was 20 mgs. per cent. The animals were divided into four groups of five animals each, so chosen that the average value of each group was 20 ± 1–2 mgs. per cent. Into each animal, 0.2 cc. of a sea-water soluble fraction of the eyestalks of *Uca pugilator* was injected, this volume being equivalent to one Uca eyestalk. Blood samples were taken from the first group one hour following injection, from the second group 2 hours after injection of this group, from the third at 3 hours after injection, from the fourth 4 hours after injection, and

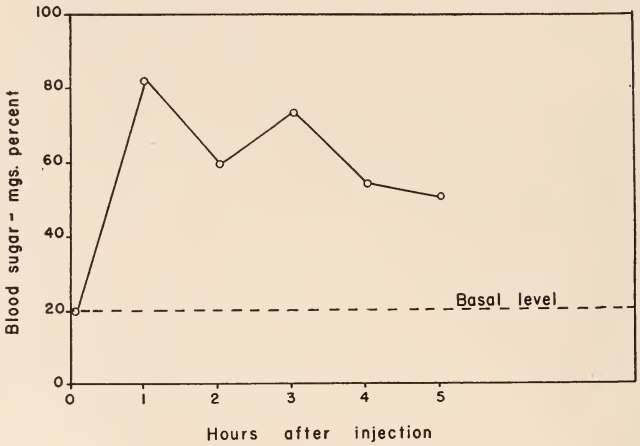


Figure 1. Curve showing the effect of a single injection of eyestalk extract (Uca) on the resting blood sugar level of the blue crab.

again from the first group 5 hours after the injection of this group. The results of this experiment are shown in Figure 1. The diabetogenic effect of the eyestalk extract is not due to reducing substances contained in the extract for, in the above experiment, the amount of extract injected into each animal contained only 1.3 mgs. per cent reducing substances calculated as glucose.

2. The Effect of Dilution on the Diabetogenic Action of Eyestalk Extracts

Eyestalks of *Uca pugilator* were ground thoroughly with a small amount of sea-water, the soluble portion decanted, and the residue re-extracted twice in the same fashion. The combined soluble portions were centrifuged, and from the super-

natant solution a series of dilutions ranging from 5 E.S.[3]/cc. of solution to 0.005 E.S./cc. solution was made. The amount of material injected into the test animals prepared as described above, was in all cases 0.2 cc. Blood samples were withdrawn from each specimen one hour following the injection. The results of this experiment are shown in Table I. It is evident that a rough agreement between

TABLE I

The effect of dilution on diabetogenic activity of the extracts

Number of animals injected	Dosage of extract	Blood sugar values—mgs. %
5	1.0 E.S.	82.4 ± 5.1
4	0.5 E.S.	59.5 ± 10.3
8	0.1 E.S.	49.5 ± 5.5
5	0.05 E.S.	47.0 ± 6.2
7	0.01 E.S.	46.4 ± 11.1
10	0.001 E.S.	41.6 ± 3.9
12	0.2 cc. sea water	24.5 ± 2.3
18	uninjected controls	20.0 ± 1.6

dosage and the resulting hyperglycemia exists, the greatest dose producing the greatest increment. However, there is little difference in the values obtained among the doses ranging from 0.1 E.S. to 0.001 E.S. The greatest response produced was a four-fold increase (1.0 E.S.) but it should be remarked that five- and six-fold increments in blood sugar values have been obtained with extracts of the eyestalks of Callinectes in the same, or even smaller doses. Injection of sea-water was without effect, the slightly higher value (24.5 mgs. per cent) for this group over the uninjected controls (20.0 mgs. per cent) being statistically insignificant. The extract was active in the lowest dosage given (0.001 E.S.) since the value obtained (41.6 mgs. per cent) represents a significant difference over either the uninjected or the sea-water injected control groups. However, some of the readings obtained with higher doses are not significant, this being due to the large standard deviation.

3. The Effect of Boiling on the Diabetogenic Effects of the Extract

Concentrated aqueous extracts of Uca eyestalks were divided into halves, one of which was placed in a water bath at 100° C. for several minutes, and the other left untreated. After heat coagulation, the boiled extract was centrifuged and the supernatant fluid diluted to the original volume before boiling. Both boiled and unboiled extracts were greatly diluted with sea-water so that each animal was injected with 0.2 cc. of a dilution containing 0.5 E.S./cc., or 0.1 E.S. total dose per animal. Blood samples were taken as usual one hour following injection. No difference was found in the activity of the boiled and unboiled extracts (Table II).

4. Localization of the Diabetogenic Activity of the Eyestalk

Four eyestalks of the blue crab were opened by a longitudinal incision, and the sinus gland of Hanström dissected out.[4] The glands were macerated in 4 cc. of

[3] The letters E.S. are used as an abbreviation of eyestalk.

[4] The position of the sinus gland was kindly demonstrated by Dr. F. A. Brown, Jr.

TABLE II

The heat stability of the diabetogenic factor

Dosage	Number of animals injected	Average blood sugar values—mgs. per cent	
		Boiled	Unboiled
0.1 E.S.	4, 4	56	53
0.1 E.S.	6, 6	61	54

sea-water and centrifuged. 0.25 cc. of the extract was injected into each of four animals, the amount being equivalent to one-fourth of a sinus gland. The eyestalks from which the sinus glands were removed were also macerated with 4 cc. of sea-water, centrifuged, and 0.25 cc. of the extract injected into each of four test specimens. One hour after injection, the average blood sugar of the group receiving the sinus gland extract was 83 mgs. per cent, and that receiving the eyestalk (minus sinus gland) extract was 30 mgs. per cent.

5. Effects of Extracts of other Tissues

Saline extracts of the hepato-pancreas of the blue crab were without effect on the resting blood sugar level. Similar extracts injected into specimens made hyperglycemic (100–120 mgs. per cent) by various means produced variable results; in two cases, a sharp fall was obtained while in the majority of cases insignificant changes were observed.

6. The Effect of Eyestalk Extirpation

Twenty-four crabs were isolated and starved for 3 days, after which time the eyestalks were surgically removed from 12 animals, the other 12 remaining as controls. The animals were then starved for another 7 days during which three sets of analyses were obtained from both normal and operated groups, on the second, fifth and seventh days following operation. The results are shown in Table III.

TABLE III

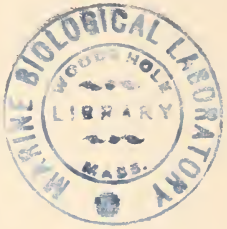
Animals	Blood sugar—mgs. per cent		
Days after operation	2	5	7
Normal (12)	22.6 ± 1.5	25.2 ± 4.5	22 ± 1.6
Operated (12)	24.4 ± 1.6	36.7 ± 4.6	40.3 ± 10.2

The blood sugar of the operated specimens tends to increase following the removal of the diabetogenic factor of the eyestalks, but the differences between the two sets of animals are not significant due to the large standard deviation. The results, however, seem paradoxical to those obtained from injection experiments, but it must be remembered that the operative injury may be sufficient to produce these high and

irregular values and thus to mask any possible hypoglycemia resulting from the removal of the eyestalk factor. This experiment should therefore be repeated with animals from which the sinus gland only is removed.

Summary

A powerful diabetogenic factor has been found in aqueous extracts of the eyestalks of crustaceans. The activity of the extracts is interspecific, heat stable, and effective over a wide dilution range. The sinus gland of Hanström appears to contain most of the diabetogenic factor.



STUDIES ON THE POSTEMBRYONIC DEVELOPMENT OF HYALELLA AZTECA (SAUSSURE)[1]

SISTER FRANCIS SOLANO GEISLER, S. S. J.

(Catholic University of America, Washington, D. C.)

Hyalella azteca is a common and widely distributed fresh-water amphipod crustacean. The genus Hyalella is the only one of the family Talitridae occurring in the fresh waters of America. The species *H. azteca* described by Saussure in 1858 from Vera Cruz, Mexico, where it was originally found among the ruins of the Aztec Indians, is widespread in North and South America, and has been cited in the literature more often under the synonyms *Hyalella knickerbockeri* (Bate) or *H. dentata* Smith.

Holmes (1902, 1903) made observations on *H. dentata* Smith to determine the mode of sex recognition, food habits, thigmotaxis, phototaxis, and reaction to pressure. Weckel (1907) discussed the synonymy of this species and gave a minute description of its characteristic external features. Embody (1911), interested in propagating amphipods as food for fishes, made a study of the distribution, food, and reproductive capacity of four common fresh-water amphipods, including *Hyalella knickerbockeri*. He states that this amphipod breeds for 152 days during the warmer months of the year and averages about 18 eggs (15 times in 152 days) per brood.

Jackson (1912) investigated the distribution and habits of this species, also its color, size, moulting, effects of starvation on moulting, breeding, locomotion, and enemies. In his paper there is no mention of temperature, and the age of the animals was judged by their size. In 1915 Phipps made an experimental study of the behavior of amphipods with respect to light intensity, direction of rays and metabolism. *Hyalella knickerbockeri* was one of the animals included in his study. Gaylor (1922) reported on the life history and productivity of this species, and recently, the effects of population density upon its growth, reproduction and survival were studied by Wilder (1940).

In the above-mentioned investigations of *Hyalella azteca* no account exists of the morphological changes that occur in its postembryonic development, a necessary preliminary to experimental studies for which this animal seems suitable. In fact, few amphipods have been so studied. The work of Sexton (1924) on *Gammarus*

[1] A contribution from the Department of Biology, the Catholic University of America, Washington, D. C. This paper, prepared under the direction of Dr. Edward G. Reinhard, is based on the author's dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The writer wishes to express her gratitude to Dr. Reinhard who suggested the problem and gave constant assistance and encouragement throughout the work. To Dr. T. von Brand and Dr. W. G. Lynn thanks are due for helpful suggestions. The assistance of Mr. Clarence R. Shoemaker, Associate Curator of Marine Invertebrates, U. S. National Museum, who graciously placed at the author's disposal his fund of information on amphipods, is likewise gratefully acknowledged.

chevreuxi is undoubtedly the most outstanding contribution to our knowledge of the postembryonic development of amphipods. Sexton raised the amphipods individually from birth and studied their complete series of moults. When her work was begun in 1909 nothing was known as to the length of life of an amphipod, its moulting periods, incubatory period of the eggs, time required to reach maturity, etc. In addition to answering these questions for several species, she found the number of growth stages from hatching to sexual maturity to be different not only in the different genera, but different even in the various species of any one genus. *Gammarus locusta* lays eggs after the twelfth moult, *G. chevreuxi* after the seventh, and *G. pulex* after the tenth moult.

With the hope of adding to the knowledge of amphipod development, and keeping in mind broader biological problems, the question of growth and differentiation of *Hyalella azteca* was investigated according to the following plan:

1. Growth, from hatching to sexual maturity, considered with respect to the number of moults and the morphological differences between the instars.

2. Correlation between the differentiation of external secondary sex characters and the development of the gonads.

Materials and Methods

The Hyalellas were obtained from Mr. Eugene W. Surber, in charge of the Fish Hatchery of the U. S. Fish and Wildlife Service at Kearneysville, West Virginia. They are descendants of a stock originally collected by Mr. Surber in a Mississippi River slough near St. Charles, Missouri.

The study of growth, from hatching to sexual maturity, was investigated according to the following procedure: Young Hyalellas were taken on the day they emerged from the brood pouch of the mother and placed in individual culture dishes. For the newly-hatched animals ordinary Syracuse dishes were used. Bits of freshwater plants, such as Elodea and Vallisneria were added to the tap water and sand in the culture dishes. When the animals grew larger and there was danger of their crawling over the edge, larger dishes were substituted. The young animals were kept in a dark room where a constant temperature of 16 to 18° C. was maintained. Cultures were examined daily, and the moults preserved separately in vials of 5 per cent formalin until complete series from birth to maturity had been collected. Niagara Sky Blue, an aniline dye excellent for chitin, was used in staining the moults, and permanent mounts were made in Diaphane.

As soon as the first formation of eggs was observed in the young developing female, a male from a stock culture of mating males was added to her culture dish. Both the date of pairing and date of egg laying were recorded. The number of moults that preceded each of these events was likewise noted. Further observation was made in regard to the development of these eggs and the number of young produced in first broods.

When males could be identified by their large gnathopods, females (from the stock culture) with ripened eggs in their ovaries were added. Females were used that had just released young from their brood pouch and had new eggs ready to be fertilized. After pairing had taken place and eggs passed into the brood pouch, the eggs were observed for development, as this was the only means of determining whether or not the males were mature.

A comparison of the moults was made to study the earliest appearance and subsequent differentiation of secondary sexual characters. Brood plate development was considered in relation to growth of the ovary. Changes in the size and shape of plates as well as the time of the appearance of hairs were recorded. In the male, gnathopod differentiation was studied in its relation to the mating period. In addition, moults were studied for antennal growth and development of dorsal teeth.

General Observations

A. Description of Hyalella azteca (Saussure)[2]

The body of *Hyalella azteca* is elongated and laterally compressed, with the first thoracic segment fused to the head. The length of the animal is 4 to 6 mm. The thorax is composed of seven and the abdomen of six segments; the telson is small and entire. The eyes are sessile, compound, and round or nearly so. Antenna I is shorter than antenna II and is without an accessory flagellum; these features being characteristic of the family Talitridae. The peduncle of antenna I consists of three joints; the first and second joints are about equal in length and slightly longer than the third; the flagellum is composed of seven to nine joints, and is about twice as long as the peduncle. The peduncle of antenna II consists of five joints, on the second of which is located the antennal gland. The two distal joints of the peduncle are elongated and nearly equal. The number of joints in the flagellum of antenna II varies from eight to fifteen. Maxillae and maxillipeds will not be described as the study of their development does not enter into this investigation.

All thoracic legs, except the first and last pairs, bear gills on the inner side of the first joint. It is of interest to note that *Hyalella azteca* has two kinds of gills, sternal and coxal. Small lateral sternal gills are located on the thoracic segments III to VII inclusive, and the coxal gills project from the inner surface of the first joint of the thoracic legs II to VI inclusive. The first two pairs of thoracic legs differ from the others and are called gnathopods; the remaining five pairs are more or less similar in structure and are termed peraeopods.

Extending from the first three abdominal segments are three pairs of pleopods, each pleopod consisting of a long basal joint and two multiarticulated, setose rami. Pleopods are employed not only in swimming, but to direct water toward the gills. As the pleopods are constantly in motion even when the animal is resting, the gills and the developing embryos in the brood pouch are always aerated. A dorsal tooth projects from the posterior edge of each of the first two abdominal segments. The three posterior abdominal segments each bear a pair of uropods. These are directed backward and fitted for springing. The first and second pairs of uropods are biramous, and the third pair is uniramous.

1. *Males*

Males are distinguished from females by their larger size and by their second gnathopods. The propodus of the male's second gnathopod is very large, and the

[2] Smith gives a good picture of *Hyalella azteca* (=*Hyalella dentata*) in *Rep. U. S. Fish Com.*, 1872–73 (1874), p. 645, pl. 2, fig. 8.

palm is subchelate, in contrast to the female's which retains its juvenile small and chelate form. The testes, located beneath the heart, are much elongated and taper at each end. The male ducts open by papillae on the ventral side of the last thoracic segment.

2. Females

In females ova can be distinctly seen and approximately counted as they lie in the ovaries which are ventral to the heart. The paired tube-like ovaries extend from the second thoracic segment to about the middle of the seventh segment. When filled with eggs, they appear to terminate abruptly at each end. The narrow oviducts open at the base of the fifth coxal plates so that when the eggs are released they are caught in a brood pouch formed by the lamellae. Only during the passage of eggs can the oviducts be seen. In passing down, the eggs lose their ovoid shape and resemble a worm pressing its way forward. Immediately upon reaching the brood pouch the eggs assume their former shape.

The mature female can also be distinguished by lamellae, which occur on the inner surface of the coxal joints of thoracic legs II to V inclusive. The edges of the lamellae, except the posterior edges of the fourth pair, are bordered with hairs that are hook-shaped at their distal end. Hairs from adjacent lamellae entangle about each other and help to hold the eggs in the pouch.

B. Observations on mating

One of the characteristic features of the Amphipoda is their habit of pairing. Smallwood (1905) observed that even the sand fleas, *Orchestia palustris,* retain the habit of pairing, a very awkward process on land. Gaylor (1922) observed that Hyalellas pair for as many as seven days before copulation. In his discussion of sex recognition, Holmes (1903) states that pairing of the animals is determined by the reaction of the animals when they collide with each other.

Pairing is followed by the shedding of the female's moult, a necessary preliminary to copulation. Normally, the oviducts are closed externally, and temporary openings are only present after the moult when the cuticle is soft. The male then deposits sperm in the brood pouch around the orifices of the oviducts, and the eggs are fertilized as they enter the pouch. It may be mentioned here that the female Hyalella is able to release eggs into the brood pouch without the influence of the male, contrary to the statement of Embody. However, as such eggs are unfertilized, they apparently disintegrate and are eventually lost.

After copulation the animals cease swimming together. When a female bearing eggs in her brood pouch is found paired it is evident that the embryos in the pouch are well developed and will hatch in a few days, and the male is to become father of the next brood. Usually the ovaries are by this time filled with ripening eggs.

At the temperature at which Hyalellas were kept, about 21 days were required for development of the eggs in the brood pouch, i.e., from fertilization to hatching. After hatching, the young remain in the brood pouch for one to 3 days. One can observe the movement of their pleopods within the mother's pouch.

When ready to emerge, the young crawl to the front of the pouch which is open. After reaching the edge, they are abruptly thrown off as the mother makes a sharp

turn in her swimming movements. If the mother is again paired, the young do not remain in the brood pouch very long; the jerky movements of the male cause them to make an early exit. Some young were seen making their exit through the floor of the brood pouch by pushing the lamellae aside and tearing the hairs.

Instars Described in Terms of Moults

A. Four stages of development

Newly-hatched Hyalellas possess all the appendages of the adult, except the secondary sexual characters. Naturally, many modifications occur during the growth process. Unlike the amphipod, *Pontoporeia affinis,* in which the young possess male characters (Segerstrale, 1937), Hyalella young may be said to resemble the adult female, for the reason that the young have slender gnathopods like the female. Later, the males are distinguished by the increasing size of the propodus of the second pair of gnathopods.

In describing the growth stages of Hyalella, the instars may be conveniently divided into four characteristic periods: the Juvenile, the Adolescent, the Nuptial, and the Adult.

Table I

First occurrence of pairing determined for 20 males and 14 females in relation to instar age

Instar	5	6	7	8	9	10	11
Males.....	0	1	2	12	5	0	0
Females.....	0	1	0	13	0	0	0

The *Juvenile Period* includes the first five instars. First instar is that period extending from the time the animal emerges from the brood pouch to the time it sheds its first moult, assuming as seems likely, that no moult takes place while the young are still in the pouch. During the first five instars the sexes cannot be distinguished. The head of the newly-hatched animal is large in proportion to the rest of its body. Growth is slow and gradual, and the first antenna adds a maximum of three joints. In size and shape the propodi of the first and second gnathopods are similar. Dorsal teeth make their appearance in the third moult. With the shedding of the fifth moult the animal ends its Juvenile Period.

The *Adolescent Period,* including instars 6 and 7, shows an increased rate of growth in the entire animal. The sexes can now be distinguished. The propodus of the male's second gnathopod is much enlarged and the palm is transverse. The animals that retain the slender propodus at this period turn out to be females (with the exception of some retarded individuals). The paired tube-like gonads become more prominent and surrounded by adipose tissue. In some of the males the digestive tube, which is beneath the gonads, curves noticeably downward in the thoracic region and is lowest in the fourth, fifth, and sixth thoracic segments. In the seventh instar some of the females show the first formation of a few small eggs in the region of the fourth and fifth thoracic segments. Small brood plates are present but hairs are lacking on their borders. Another joint is added to the first antenna, and the fourth joint has again elongated and is ready to constrict.

The *Nuptial Period* designates the eighth instar during which the animals usually pair for the first time (Table I). Males pair almost as soon as the moult is shed, while the females generally pair toward the end of the period when the eggs in their ovaries have fully matured.

The animals are very much larger and the propodus of the male's second gnathopod is greatly enlarged with a distinct subchelate palm. One or two joints are added to the first antenna. The female's lamellae are turned back at the edges and a few hairs are present in the eighth moult which normally marks the end of the Nuptial Period.

The *Adult Period* occurs after the eighth ecdysis. Eggs are released into the brood pouch formed of fully developed lamellae bordered with hooked hairs. The eggs are fertilized by the sperm previously deposited around the orifices of the oviducts. Moult IX has an additional first antennal joint. When the ninth moult has been shed the animals have not attained full size. Growth continues beyond the tenth instar, but detailed observations were not carried on beyond this point.

Table II shows the duration of instars in days of the animals in Brood C.

TABLE II

Sample record showing duration of instars in days, Brood C, 12 individuals, emerged 12/8/42

Animal Instar.....	1	2	3	4	5	6	7	8	9	10
♀C1.....	8	6	13	12	11	10	16	(died)		
♂C2.....	8	17	5	10	15	11*	17	19		
♀C3.....	8	5	9	9	10	9	7	10*		
♀C4.....	9	26	12	10	8	12*	22			
♂C5.....	9	14	7	10	7	9	20	16*		
♂C6.....	9	13	11	8	9	9	10	12*	30	
♀C7.....	9	14	9	9	9	9	9	12*	25	
♂C8.....	10	14	9	8	9	10	12*			
♀C9.....	10	16	15	4	8	9	11	16*		
♂C10.....	11	15	10	6	9	10	9	24*	23	
♂C11.....	11	15	10	8	10	8	10	10	18*	
♂C12.....	12	12	8	10	9	9	12	17*		

The asterisk indicates the instar during which the animals paired for the first time.

B. General growth rate

At first growth is slow, moults I and II appearing to be the same size. The duration of the second instar is quite long; this is evidence of slow growth. In Figure 1 the time in days between moults is graphically represented for seven broods. Instars 3, 4, and 5 are short and during this period growth is rapid. Following moult V, differentiation of secondary sexual characters sets in. As the animals age they shed their moults less frequently. It is a known fact that young animals moult more frequently than do adults. Adult females moult more often than do adult males.

Body size in terms of moult length was determined for three representative males and two females, the measurements of the moults being made from behind

the head to the tip of the telson. The averages are seen in Figure 2. This graph shows that moults II, VI, and VII are larger in the females studied than in the males. However, general observations indicate that after attaining maturity the male grows at a faster rate than the female and as adult is characteristically larger

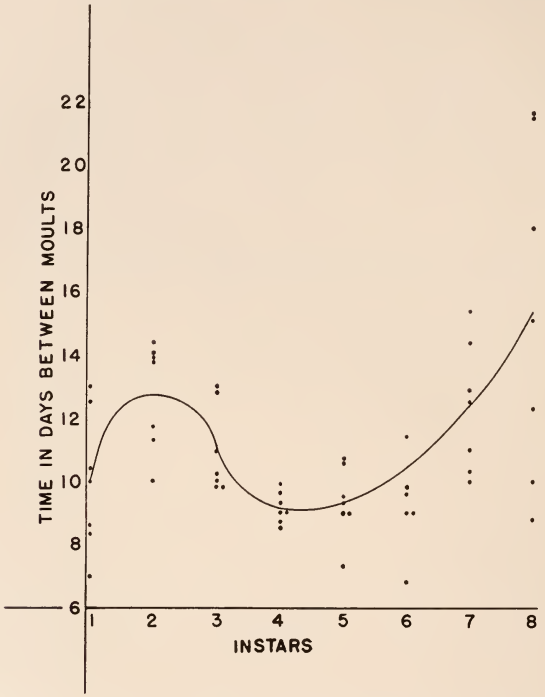


FIGURE 1. Time in days between moults measured for seven broods.

than the adult female. This does not appear on the graph since detailed studies in this case did not go beyond the eighth instar.

C. Growth of the antennae

Newly-hatched Hyalellas have six joints in the first antenna. The three proximal joints constitute the peduncle, and the distal three the flagellum. During the process of growth the number of peduncular joints remains constant while the flagellum develops four to six new joints. The actively growing region ("growth center") is the first proximal joint of the flagellum. Growth can readily be studied through the series of moults from any one animal. A number of series were studied

to compile the following table which refers to number of joints in the entire first antenna:

Moult	I.	6 joints; No. 4 is long.
Moult	II.	6 joints; No. 4 is long. Or 7 joints with No. 4 short and lacking setae. The constriction has just occurred, and setae which are normally found on the distal edge of each joint, have not yet developed.
Moult	III.	7 joints; No. 4 is short and lacking setae.
Moult	IV.	7 joints; No. 4 is long, and has setae. Or 8 joints with No. 4 short and lacking setae.
Moult	V.	8 joints; No. 4 is long, and has setae. Or 9 joints with No. 4 short and lacking setae.
Moult	VI.	9 joints; No. 4 is long, and has setae. Or 10 joints with No. 4 short and lacking setae.
Moult	VII.	10 joints; No. 4 is long, and has setae.
Moult	VIII.	11 joints; No. 4 is long, and has setae. Or 12 joints with No. 4 short and having setae.
Moult	IX.	11 joints; No. 4 is long, and has setae. Or 12 joints with No. 4 long and having setae.

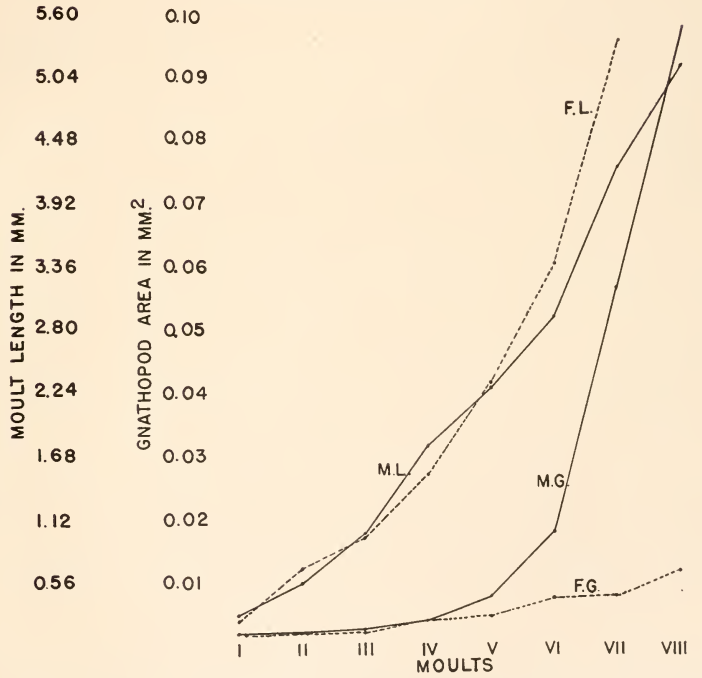


FIGURE 2. Growth in length of body of *Hyalella azteca* plotted against growth in area of gnathopods (propodus and dactylus). M.L.—length of male moult. F.L.—length of female moult. M.G.—area of male gnathopod. F.G.—area of female gnathopod.

In the early stages of development growth is relatively slow. With the shedding of two moults only one joint is added. From Moult V onward, joints are added at the rate of one per moult. Plate I is a representative series of drawings from moults shed by the animal D8. The outside view of the right first antenna is drawn to scale.

It cannot be said that any particular moult always has a certain number of joints, because the growth rate varies in different animals. Sometimes in the same moult one of the first antennae may have one joint more than the other first antenna (Pl. III). In such a moult it will be noted that in the one case the first proximal flagellar joint has lengthened and constricted, and in the other case, the corresponding joint has lengthened; due to a slower rate of growth, it has not yet constricted. In general, however, the number of flagellar joints is helpful in ascertaining the age and instar of the animal. An animal with a six-jointed first antenna cannot be beyond its third instar; if the animal has nine or ten joints, it is not yet mature, but is passing through one of the adolescent instars.

Second antennae—method of multiplication

As in the case of the first antenna, the "growth center" or region of most active growth of the second antenna is located in the first proximal joint of the flagellum. There are five joints in the peduncle and therefore the first joint of the flagellum is No. 6.

Moult I has a total of nine joints and this number does not increase in the second and third moults, although joint No. 6 elongates preliminary to constriction. Moult IV has an additional joint. In moults V to IX the number of joints steadily increases from 11 to 15.

In *Hyalella azteca* only one region of growth could be detected in the antennae, but in some amphipods more than one growing region is present. Segerstrale (1937) found this condition in the male *Pontoporeia affinis*. In the beginning, only the proximal joint of the flagellum divided, but in the third last stage, four of the more distal joints divided, and in this same stage the proximal joint divided three times. In the stage before the last, 15 proximal flagellar joints divided, so that the last stage of the male has a flagellum of 41 joints. The female of this species is distinguished by a shorter flagellum. Only the proximal joint divides. In the stage before the last there are ten joints, and the last stage shows no increase.

In *Gammarus chevreuxi* Sexton (1924) found that the proximal joint of the first antenna divides, usually into two portions, and the distal one which is the new joint she terms the "undivided primary joint." At the next moult the undivided primary joint elongates and divides into two portions, the distal part or true primary joint and the proximal portion which may be called the secondary joint. The process is repeated in each stage, the last formed primary joint subdividing and giving rise to a secondary joint; the formative zone dividing and giving off one or

PLATE I

Development of first antennae is shown above. The right first antenna is drawn to scale in outside view from the first eight moults shed by one animal. Arrows indicate growth pattern, i.e. the manner in which successive new joints are added by division of the fourth joint. Development of propodus and dactylus of first gnathopods of a female is shown below. Drawings made to scale from the first eight moults shed by a female.

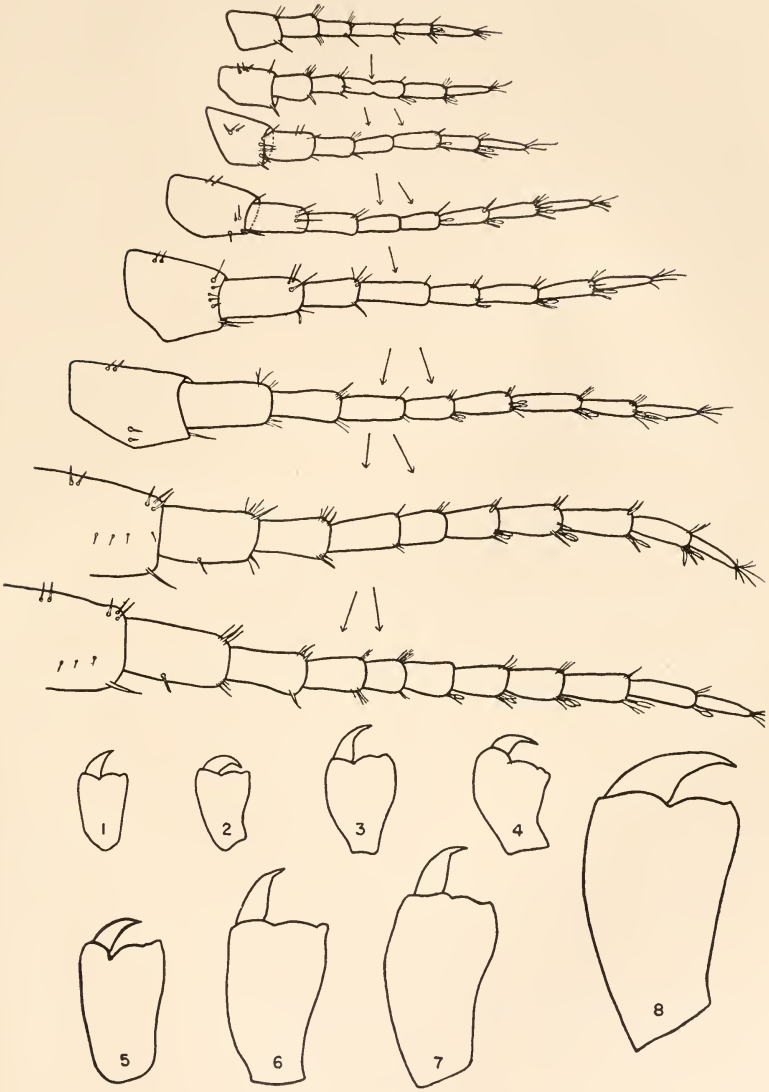


PLATE I

more undivided primary joints. Sexton also mentioned that sometimes more than one primary joint may be formed in the older animals. In *G. chevreuxi* the second antenna has a shorter flagellum than that of antenna I, and is slower also in the rate of growth.

Growth of the first antenna of *G. chevreuxi* is more rapid than that of *H. azteca*, for in moult I of the former there are seven joints in the antenna and in moult VIII of the same animal there are 23 joints. In moult I of *H. azteca* there are six joints in the first antenna and in moult VIII there is a maximum of 12 joints.

While the second antenna of *G. chevreuxi* is slower in its rate of growth than the first antenna, the rate of growth in the second antenna of *H. azteca* is about equal to that of its first antenna for six additional joints are usually present at the eighth moult.

Development of Secondary Sexual Characters

A. Gnathopods

In the early instars the propodus of the first and second gnathopods in both sexes is approximately the same size, but distinctly different in form. The palm of the propodus of first gnathopods is transverse and concave at the middle. Although this propodus increases in size with the age of the animal, it does not alter in form in either sex. A series of propodi of first gnathopods of a female, C13, is shown in Plate I. These were drawn to scale from the mounted moults of the animal. The first gnathopods of the male have similar propodi.

In contrast, the second gnathopods become sharply differentiated as the sexes mature. In both males and females the palm of the propodus of the second gnathopods is chelate during the juvenile period, and in the female the juvenile form is retained throughout life, even after the propodus has become long and slender. The complete series of propodi of second gnathopods of C13, also drawn to scale, are shown in Plate II and indicated by the letter "F."

After the fifth moult, the propodus of the male's second gnathopod gradually changes both in size and form. The palm loses its chelate form and gradually becomes transverse. From about the seventh moult on, the palm assumes a pronounced subchelate form. The dactylus of the male curves more strongly to fit the changing form of the palm. In Plate II the series of propodi indicated by the letter "M" are from male second gnathopods. All are from the male C12, except No. 3, No. 5, and No. 6 which are from the male B1. Moults III and V of C12 were badly torn, and moult VI was unusually small and not representative of a typical sixth male moult, so in the drawing the propodus from B1 was substituted.

Hundreds of male moults had been collected in an attempt to secure a complete series from one animal. Although Hyalellas do not eat their moults, they sometimes tear them badly in the process of moulting. In many cases, moults that had been preserved were found to lack both propodi from the second gnathopods, the very feature that was most essential to this study. In moulting, the male has to pull his immense propodus through the narrow carpus and in the struggle the

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Plate II

Development of propodus and dactylus of the second gnathopods. Drawings made to scale from the first eight moults of a female (F) and male (M).

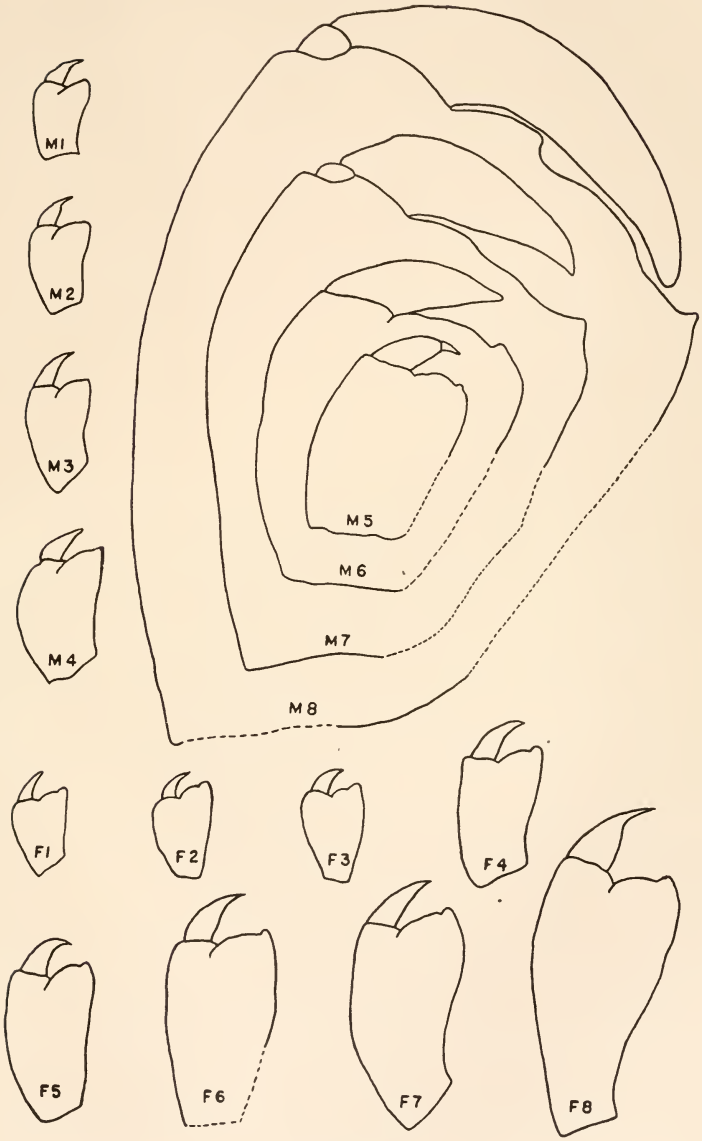

PLATE II

propodi are often torn off. In nearly every propodus that is retained the sternal border is torn.

To obtain some idea of the increase in area of the propodus of both males and females, series of drawings to scale were made, and the actual area of the gnathopod was calculated from these drawings. A complete series of propodi from one female was used, but as there was no complete series of male propodi, an average obtained from the propodi of three males was used in determining area. The data are presented in Figure 2.

The propodus of the male in moult V is 4.7 times the area of the same propodus in moult I; in moult VI, it is 11.8 times, in moult VII, 37 times, and in moult VIII, 64 times. The female propodus shows a gradual increase; in moult VIII it is 8 times the area of the same propodus in moult I (Pl. II).

In *Hyalella azteca* the gnathopods differentiate as external secondary characters before the animals are sexually mature. When males could be identified by their gnathopods as early as the sixth instar, mature females with ripe eggs in their ovaries were added. At this stage the males were not mature, because they did not pair. When those same females were removed and placed with mature males from the stock culture, pairing immediately took place and was followed by copulation.

After the young males shed their next moult and were then in their seventh instar, mature females were again added. In a very small percentage of cases pairing took place. In general, pairing took place after males had shed seven moults. The fact that young hatched from those eggs shows that they were fertilized. Although males may be regarded as sexually mature in their eighth instar, in five cases males first paired during the ninth instar (Table I). In these instances the females that were added during instar 8 may not have been in the right physiological condition for mating.

If we consider pairing as an indication of maturity, both sexes are normally mature in the eighth instar. On the other hand, if the instar during which ova deposition occurs is taken as the first sign of sexual maturity then the female is mature in the ninth instar, because her eighth moult must be shed before eggs are released. The male, in the latter case, may be said to mature one instar before the female, because he is capable of fertilizing eggs immediately after pairing and before his eighth moult is shed.

The first gnathopods of the male Hyalella are employed in carrying the female and are structurally adapted for this purpose. The fact that the second gnathopods enlarge when the male approaches maturity seems to indicate that they are necessary for sex recognition. Males need them to actively resist each other, and females in lacking them are not able to resist being carried, as described by Holmes (1903). Sexton observed that *G. chevreuxi* male carries the female with his second gnathopods, and Heinze (1932) stated that *G. pulex* male uses his first gnathopods for this purpose.

After reaching maturity, the male continues to grow, and the second gnathopods increase in size proportionately, until the maximum adult form is reached.

B. Lamellae

Lamellae, characteristic of adult females, are entirely lacking in the young animals. The sixth moult of females has small, incompletely developed lamellae; the

seventh has them slightly enlarged. In moult VIII the edges of the lamellae are turned backward and bordered with a few scattered hairs. Moult IX contains the fully developed lamellae bordered with hairs; this is the first adult moult of the female.

The hairs differ from the long, straight hairs found in Gammarus and certain other amphipods. In Hyalella, as in other members of the family Talitridae, the basal portion of the hair is slightly enlarged, and the distal end is swollen and curved around in the shape of a hook. The hooked ends join with hairs from opposite or adjacent lamellae to help hold the developing eggs in the pouch (Pl. III). When eggs are first deposited in the pouch, opposite lamellae overlap each other. In later stages, when the embryos are large, the lamellae are fully stretched out and only the hairs cling together. Perhaps the increased weight or the movement of the young push the lamellae out to their full extent. Small, young females generally have fewer eggs than older females which are larger, and the size of the lamellae is in proportion to the size of the animal. Four to 13 young were produced in first broods.

The fourth pair of lamellae is smaller than the three anterior pairs and the posterior margin, which is slightly chitinized, is curved upward. Hairs are lacking along that same border. Due to the upward curvature of the lamellae, it may be that hairs are not necessary. A few spines are located on the external side of the curved portion.

Development of hairs is in some way correlated with maturing of the eggs, for they appear and function for the first time when the first eggs are released.

The development of lamellae in Hyalella is similar to that of other amphipods. Sexton reported that in *G. pulex* where the cuticle is firmer and more substantial than in *G. locusta* or *G. chevreuxi,* she has been able to trace brood plates in four growth stages preceding maturity. First they appear as minute, rounded leaf-like plates with margins entire; in the next moult they have lengthened and increased to three times the size; rudimentary hairs make their appearance on the next moult, and in moult X (the moult after which eggs are laid) brood plates are very large, chitin is hard, and rudimentary hairs more numerous. In the next stage when sexual maturity is reached, the brood plates are fully developed with long fringing hairs. In *G. locusta* and *G. chevreuxi* Sexton traced brood plate development in three stages before sexual maturity.

Heinze, too, from his study of *Carinogammarus Roeselii* Gerv. concluded that the first anlagen of oostegites appears several moults before the female becomes mature. In fact, he found the first pair of lamellae on the third segment when the animal was 4.5–5 mm. Heinze does not mention the instar of the animals, but gives their length in millimeters. When the female is 5–5.5 mm. long the four pairs of brood plates are present, the fourth pair on segment 6 being the narrowest. The closer the female approaches maturity, the larger the brood plates become. In stages 7.5 mm., 8 mm., 8.5 mm. the hairs are still lacking, and in the next stage, bordering of the brood plates with hairs precedes copulation. In *G. pulex* Heinze found similar conditions, the first pair of brood plates appearing when the animal is 4.5 mm. But corresponding to the larger size of the female, the oostegites in their development were more advanced.

Segerstrale reported that the first anlagen of brood plates in *Pontoporeia affinis*

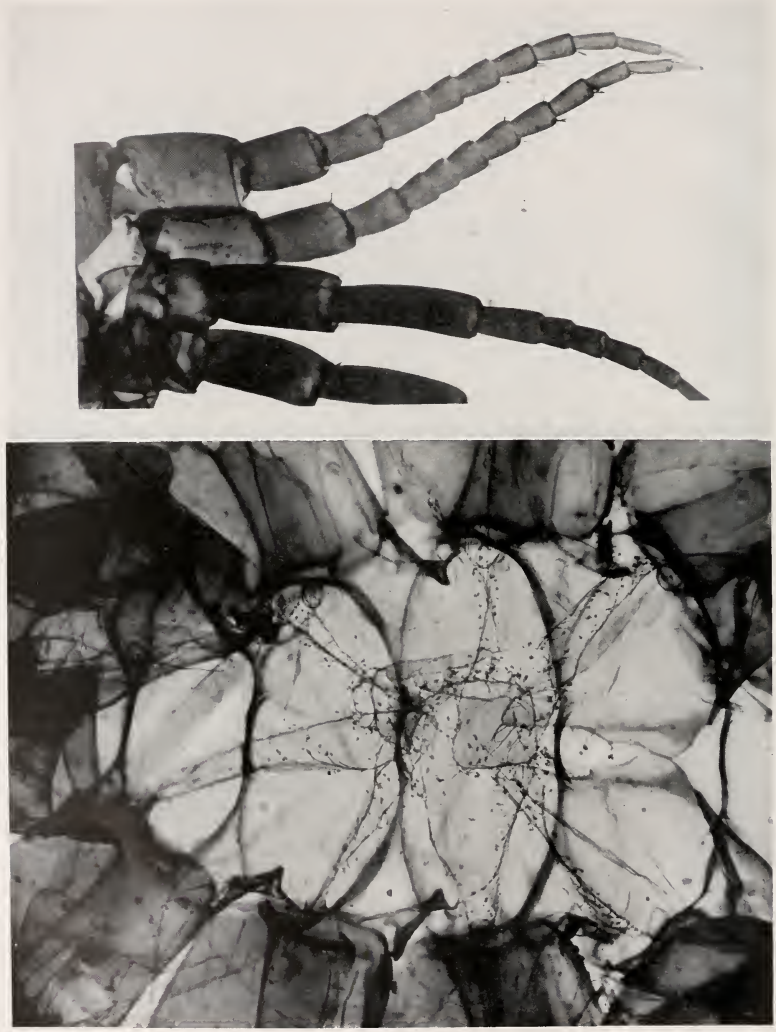

PLATE III

FIGURE 1. A case of unequal growth of the first antennae. A constriction has appeared in the first proximal flagellar joint of the right antenna, while the same joint in the left antenna has elongated but not yet constricted. × 68.

FIGURE 2. Photomicrograph of female moult to show brood plates with interlocking hairs as seen from within. × 68.

occurred in females that were 5.3–5.5 mm. in body length. They appear as small rounded structures which expand by degrees so that in the stage before the last they show approximately the final form. The enlargement of the area occurs in the greatest degree only during the last moult.

Retarded Individuals

During the course of this investigation, it soon became apparent that all the animals did not mature at the same time. This was first noticed in the animals that were raised individually. In the eighth instar the animals generally paired, showing that they were sexually mature. About one or two in some broods failed to pair at this time, due to their immature condition. For the present, since their precise nature is unknown, these slow-developing animals will be termed "retarded individuals." At a stage when their siblings could readily be distinguished by their gnathopods, and also by eggs in the case of the females, these retarded individuals, while possessing the slender gnathopods of the female, lacked visible eggs. Retarded individuals are smaller than the rest of the animals in the same brood and grow at a slow, steady rate. At a much later stage some of them gradually developed male gnathopods and eventually paired. Two others after the tenth moult and therefore during the eleventh instar, developed eggs and later paired. One of them lost its first brood of eggs, but its second brood, which had been fertilized by a retarded, but now mature male, developed into young in the normal length of time. However, none of the young reached maturity.

Two retarded individuals, aged 67 days (the rest of their brood had matured at 40 days), when sectioned and stained, showed a few very small eggs in their gonads. Another, having female gnathopods but lacking eggs, was dissected. It was found to have large, normal brood plates that lacked hairs.

It is possible that the retarded individuals are similar to the intersexes described so completely by Sexton. A thorough study of them, outside the scope of the present investigation, would have to be made to determine their exact status. It would be interesting to learn whether a deficiency, hormonal or nutritional, prevents maturation of the gonads at the normal time, whether their genetic constitution warrants a later maturation, or whether retardation may be caused by a state of depression induced by unfavorable external factors. A study of this special problem is now under way.

Summary

Hyalella azteca grows well under laboratory conditions. Like other amphipods, the frequency of its moulting depends upon several factors, chief of which is temperature. At low temperatures ecdysis is delayed. Frequency of moulting in developing animals is no indication of sex, as it occurs at varying time intervals in both sexes. The young moult more often than do the adults. In reaching the time for first pairing, the number of moults is more important than the age, in days, of the animal. First pairing generally occurs in the eighth instar.

In general, growth at first is slow. After the secondary sex characters first become apparent (Instar 6) growth becomes more rapid.

Centers of more active growth are present in certain regions, such as the second gnathopod of the male, and the proximal joint of the flagellum in both pairs of

antennae. In the female the fifth thoracic segment appears to be a "growth center," for eggs begin forming in this region. No doubt, there are regions of active growth throughout the body, but the above are the most prominent.

Animals during any instar are not the same size. Males particularly vary in size, although they may mature after the same moult. Retarded individuals, animals that pair much later than the normals, are fairly common. It is possible that some of them never become sexually mature.

The external secondary sex characters, lamellae and gnathopods, make their appearance several moults before the animals pair. Lamellae grow gradually, and reach their full size and become bordered with hooked hairs when the first eggs are released.

First gnathopods in both sexes are similar in size and form. Second gnathopods of the female retain their juvenile form, although they increase in length. Second gnathopods of the male develop a very large propodus with a strong dactylus and the palm of the propodus changes from chelate to transverse and then finally to subchelate.

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CHANGES IN WEIGHT AND WATER CONTENT DURING THE LIFE CYCLE OF THE WOOD-EATING BEETLE, PASSALUS CORNUTUS FABRICIUS

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Introduction

That relative humidity and water relations in general are important in the lives of insects has been well established. The literature on the subject is voluminous. Excellent reviews by Buxton (1932), Mellanby (1935), and Johnson (1942) discuss insect water relations from various points of view, such as saturation deficiency and longevity, evaporation of water, the humidity of the environment, etc. Others (e.g. Johnson, 1937; Slifer, 1938) have studied the mechanism and rate of water absorption in the insect egg. Ludwig (1931), in reviewing the literature on the subject of changes in weight and water content during metamorphosis of insects, calls attention to the fact that in different species entirely opposite phenomena may take place at corresponding stages in development. To give one example: Bodine and Orr (1925) observed that in Drosophila a marked *increase* in weight occurred on emergence of the adult, while Ludwig (1931) found that during the metamorphosis of the Japanese beetle from larva to adult there was a *loss* of weight amounting to half the maximum larval weight. In both cases the changes in weight were attributed mainly to changes in water content. Thus it would seem that no hard and fast rule can be laid down that will apply to all insects; the story must be worked out independently for different species living under different conditions.

In this paper an attempt is made to establish the normal weight and water content of a beetle, *Passalus cornutus,* at all stages in its life history from egg to adult under stable conditions. A study of the ranges of tolerance to humidity and temperature are left for a later paper. Among the beetles there are few, if any, records of the changes in water content of a species throughout its whole life cycle. Ludwig's (1931) studies of the weight changes and water relations of the Japanese beetle larva, pupa, and adult offer the most satisfactory comparison to the conditions found in Passalus, although the habitat and duration of the life cycle of the Japanese beetle and of Passalus differ markedly.

Several factors make Passalus a convenient insect for study: large size, abundance, ease in obtaining, large size of eggs, and short life history. Passalus is a wood-eating beetle which spends practically all of its life in decaying hardwood logs. The adult beetles make extensive tunnels in the logs; and here the eggs are laid, and larval, pupal, and adult life is lived. The eggs are often deposited in an offshoot of a tunnel, out of the way of the main line of traffic. Although not all eggs of one female are laid at the same time, all her eggs are laid in the same place in the runway. Always they are on, and surrounded by, an abundance of moist,

finely chewed frass prepared by the adults. Passalus is a social beetle. The adults protect the developmental stages, prepare frass for the larvae, and build cocoons for the pupae. The developmental cycle is completed in a relatively short time for so large a beetle. In the vicinity of Durham, N. C., eggs are laid in late May or June and new adults appear in August.

Only hardwood logs that have a high percentage of moisture are inhabited. In fact, the whole life cycle must of necessity be completed in a moist environment. Data at hand indicate that eggs will not hatch nor larvae develop except when in contact with moisture. Even the adults cannot live at low humidities, and the adults are more hardy in this respect than are the eggs, larvae, and pupae.

Materials and Methods

Passalus eggs of known age were obtained in the laboratory in the following manner. Laying females were brought in from the woods and placed in finger bowls with some of the frass from the logs. One female was placed in each finger bowl. The dishes were covered and kept in a dark cabinet. Each morning the frass was examined for eggs. When first laid the eggs are bright red in color and are not difficult to distinguish from the frass. As Passalus seldom lays more than two eggs in a 24-hour period, and as all females brought in do not lay in the laboratory, it takes a great many beetles to obtain a sufficient supply of eggs for experimental purposes. The eggs were placed in petri dishes containing either moist filter paper or moist frass. Volumes of the eggs were determined by the formula $V = \frac{4}{3}\pi a^2 b$, where a equals the shorter semi-axis and b equals the longer semi-axis. The calculations are probably not absolute, for all eggs are not perfectly ovoid, but volumes computed by this formula are relative and give very satisfactory comparisons. Measurements of the eggs were made to the nearest tenth millimeter with a vernier caliper. In all, several thousand eggs have been used in various experiments over a period of several years.

All stages in development were kept in an insulated cabinet at 27° C. Larvae, prepupae, and pupae were kept in large covered dishes with an abundant supply of frass and moisture. Larvae of different ages were kept in separate dishes to prevent the larger larvae from injuring and eating the smaller. Adults were not kept with the developmental stages. All weighings were made on a chain-o-matic balance. To determine water content, from 15 to 25 eggs of each age group were dried in a vacuum oven at 98° C. until the weights became constant.

Observations

Changes in weight, volume, and water content of the eggs

Figure 1 shows graphically the close correlation between weight and volume of Passalus eggs at different age levels. The data for these curves were obtained from material collected during a single season and kept under similar conditions. About 350 eggs are represented. All were weighed and measured individually, some each day, others every other day. That the increase in weight is due to an increase in water content is clearly shown in the parallel curves of Figure 2. The eggs represented here are not the same as those used for plotting the curves of Figure 1 and were collected in a different season. In each age class 15 to 25 eggs were weighed

collectively, dried, and reweighed. The curves indicate that there is a slight decrease in dry weight as development proceeds.

Passalus eggs are large as insect eggs go, and are ovoid in shape. When first laid they measure approximately 3.20 mm. × 2.50 mm. and increase in size to about 3.80 mm. × 3.20 mm. at time of hatching. Newly laid eggs weigh between 10.0 and 12.0 mgs.; at time of hatching between 18.0 and 21.0 mgs. There is of course considerable individual variation in size and weight of the eggs. The amount of water in the eggs increases from 7.25 mgs. in newly laid eggs to over 15 mgs. in those ready to hatch. In per cent the water content increases from 68 to 83. Weight, volume and water content do not show marked changes for the first five

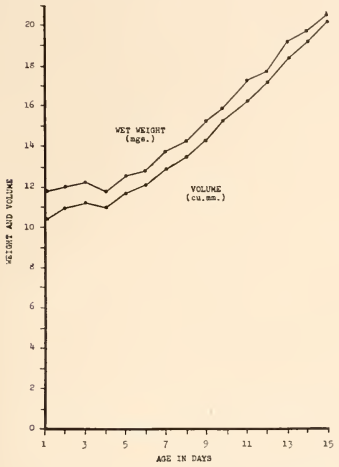

FIGURE 1. Changes in wet weight and volume during development of the eggs of *Passalus cornutus*.

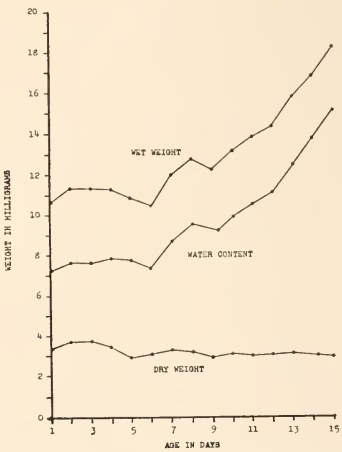

FIGURE 2. Changes in wet weight, dry weight, and water content during development of the eggs of *Passalus cornutus*.

days of development; but following this initial period of relative stability, weight, volume, and water content increase rapidly and continue to do so up to the time of hatching, which at 27° C. requires 15 days. Changes in color accompany the changes in weight, volume, and water content. Newly laid eggs are bright red. This gradually turns to a reddish-brown during the first week of development, then to a brownish-green, and within a few days of hatching to dark green. The significance of the color changes has not been determined.

Changes in weight and water content of larva and pupa

There are three larval instars. In the laboratory the first lasts about 16 days, the second about 13 days, and the third (including the prepupal stadium) about 28 days. Near the end of the third instar the larvae cease feeding and wander about

for a few days seeking a place to pupate. Entrance upon the prepupal stadium is gradual and is determined with difficulty. As soon, however, as the prepupae become sluggish and take on their characteristic white, fatty appearance, they can then be readily distinguished from the active, bluish larvae that are still feeding. The prepupal stage lasts 5 to 6 days.

On hatching from the egg the larvae vary in length between 8.5 mm. and 11.5 mm. and weigh between 18.0 mg. and 23.0 mg. Little or no food is taken for the first day or so, but after feeding is well under way—in 24 to 36 hours—the color changes from white to bluish and the increase in size and weight is rapid. There is great individual variation, but the weight of a larva may increase to as much as

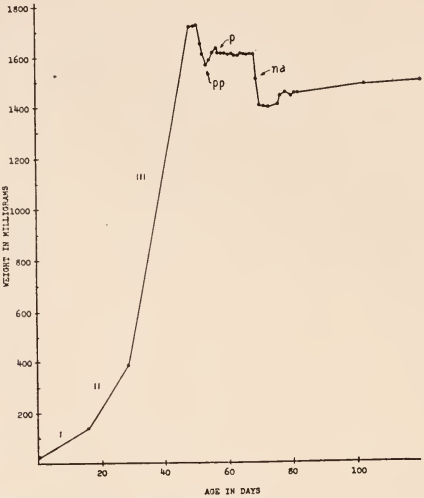

FIGURE 3. Changes in weight of *Passalus cornutus* from larva to adult. I, II, III, the three larval instars; pp, beginning of prepupal stage; p, beginning of pupal stage; na, newly transformed adult.

140 mg. by the end of the first instar, 400 mgs. by the end of the second instar, and 2000 mgs. by the end of the third instar. Thus the weight may increase a thousandfold in less than 2 months. The weight falls slightly after the late third instar larvae cease feeding and enter the prepupal stage; it rises slightly just before pupation begins; and again falls off, this time abruptly, on emergence of the adult. The weight of the adult is about five-sixths that of the greatest weight of the larva. Individual weight records were kept on many larvae raised in the laboratory and carried through to adulthood. A typical weight curve is given in Figure 3. Among the adults, as in all other stages, great individual variation in weight occurs. There is no sexual dimorphism, but the average female is larger and about 12 per cent heavier than the average male. The average weight of 27 old (not newly

emerged) males was found to be 1478 mgs.; and of 35 old females, 1686 mgs. The range in weight of males was from 977 mgs. to 1740 mgs.; of females, from 1390 mgs. to 1972 mgs.

Newly hatched larvae contain about 85 per cent water. As soon as the larvae begin feeding the water content rises to about 90 per cent. This percentage is maintained until the larva ceases feeding and enters the prepupal stage when it falls to 88 per cent. There is then a steady increase in percentage of water until a high of 93 per cent is reached in the late pupal stage. The changes in water content in the prepupa coincide with the changes in weight, but in the pupa the weight remains constant while the percentage of water increases. A loss in dry weight is apparent as the internal tissues undergo histolysis. On emergence of the adult the per cent of water does not fall abruptly to the low level of old black adults, but declines gradually. The total weight of the adult, however, remains essentially constant so that the gradual decrease in percentage of water is explained by a gradual

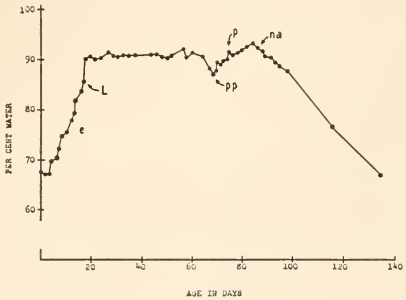

FIGURE 4. The water content, in per cent, throughout the life cycle of *Passalus cornutus*. e, egg; L, newly hatched larva; pp, beginning of prepupal stage; p, beginning of pupal stage; na, newly transformed adult.

increase in dry weight. The red color of newly emerged adults persists for several weeks and may last for several months, but as the red gradually gives way to brown and the brown to black, the percentage of water falls until it finally reaches the level of 67 per cent for old adults. Twelve adults a month after emergence were found to still contain as much as 76.5 per cent water. No appreciable difference in the water content of males and females was observed.

A curve of the changes in percentage of water content from egg to adult is shown in Figure 4.

DISCUSSION

That Passalus eggs should absorb water is not surprising for this phenomen has been observed in other insect eggs. Bodine (1929) was among the first to show definitely that the increase in weight of an insect egg is due to an actual increase in water content. Slifer (1938) has given a brief history of our knowledge of this subject and in her own experimental work has demonstrated the manner in which

water is absorbed by the grasshopper egg. She has shown that the ability to absorb water coincides with the formation of a special structure at the posterior end of the embryo. This she has designated as the hydropyle. In the eggs of *Melanoplus differentialis* there is no water absorption for the first 6 days of development (until the hydropyle has formed); then there follows a period of gradual increase in water content which lasts until several days before hatching, when the eggs become turgid and water absorption ceases. The shape of the weight curve presented by Johnson (1937) for Notostira indicates that the pattern of water absorption in the eggs of this insect is similar to that of the grasshopper: there is a lag of a few days followed by a rapid increase in weight (and water content); then a marked decline in the rate of increase occurs several days before hatching.

The curves of increase in wet weight and water content of Passalus eggs are similar to those of the grasshopper and Notostira except in one particular. In Passalus eggs water uptake begins about the fifth day, as in the grasshopper egg, but unlike either the grasshopper or Notostira the absorption of water continues up to time of hatching. The curve does not level off a few days before hatching time as in these other insects. If present at all, the "leveling-off period" must be very brief in Passalus eggs. Failure to demonstrate it cannot be explained by lack of data, for none of the many eggs, whose individual daily records of weight and volume were kept from time of laying to hatching, showed leveling-off in either weight or volume.

There is evidence to indicate that a hydropyle is formed in Passalus eggs. In fixed eggs a small ring is apparent at the posterior end of the egg about the fifth to sixth day of development, and its appearance can be correlated with the beginning of water uptake. Studies are in progress on the histological structure of the hydropyle of the Passalus egg.

As is to be expected the curve of egg volume parallels the curve of wet weight. The exact age of the egg cannot be determined by volume alone because of the large individual variations in the eggs. One can, however, estimate with a fair degree of accuracy whether an egg of unknown age is in its first, middle, or last third of development. Size and color together make fair criteria for roughly determining the ages of eggs found in logs.

The eggs practically double in wet weight, volume, and amount of water during their development. The contentions of Bodine (1929) and of Johnson (1937) that the gain in weight is due to water uptake are supported. Johnson (1937) has suggested that there is actually a decrease in dry weight of the eggs as development proceeds. It seems probable that this may be true, for in Passalus eggs there is a slight but noticeable downward trend in dry weight. However, at least in Passalus, a larger number of eggs should be used before drawing a definite conclusion on this point. The loss in dry weight is gradual and is probably caused by the transformation of the large amount of yolk into food materials and protoplasm.

In Passalus, while the curves of weight and water content of larvae, prepupae, and pupae follow the same trend as found by Ludwig (1931) for the Japanese beetle, some deviations worthy of mention occur. The time spent in larval stadia is much shorter in Passalus and growth is much greater and much faster. It is not uncommon for larvae to make a 10,000 per cent increase in weight in less than 2 months. Evidence indicates that under natural conditions in the logs in the forest,

where temperatures often rise above laboratory temperatures, growth is even faster than reported in this paper. The water content of the larva is definitely higher in Passalus. It averages about 90 per cent in contrast to 78 per cent in the Japanese beetle larva. In both larvae a drop in weight occurs in the prepupal stage. In Passalus, however, the fall in weight begins as soon as the larva stops feeding and before it definitely enters the prepupal stage and is followed by an increase in weight before the pupal stage is reached. The increase in per cent of water in the prepupa and the pupa is readily accounted for by the degenerative changes of the larval tissues which take place in these stages during metamorphosis. The pupae, especially, are extremely delicate and in their natural runways would not last long were it not for the heavy protecting cocoon built around each by adults. In the late pupa the water content reaches a high of 93 per cent. This is above that of the larva. In both the Japanese beetle and Passalus a sharp drop in weight is apparent on emergence of the adult. In Passalus, however, the decline in weight is not nearly so great as in the Japanese beetle and sometimes amounts to no more than one-eighth the maximal larval weight, although usually it is about one-sixth. This is in marked contrast to the Japanese beetle, whose loss in weight amounts to one-half the maximum larval weight. Evidently the decline in the per cent of water in newly emerged adults of Passalus is more gradual, and coincides with the gradual increase in dry weight. Sclerotization is slow. New adults are very soft and remain within the cocoon for several days before venturing forth to compete with other members of the colony. The slow increase in dry weight is correlated with the slow and gradual changes in color and in degree of sclerotization that the adults undergo. The change from pink to red to brown to black usually requires a month or more, but may require several months. Eventually the beetle becomes black. When it does so the water content has reached the level of 67 per cent common to the old adults and the integument has become thick and hard. Thus, in percentage, the water content of the adult Passalus eventually gets back to its starting point in the egg.

Summary

The changes in weight and water content from egg to adult have been shown for the wood-eating beetle, *Passalus cornutus*.

In the egg little change occurs for the first 5 days of development; then the weight of the egg, the volume of the egg, and the amount of water increase steadily until hatching. The increase in weight is due to water uptake. As weight, volume, and water content increase the color of the egg changes from bright red to dark green.

During larval life, which in the laboratory at 27° C. lasts approximately two months, the weight may increase more than 10,000 per cent. A slight fall in weight occurs when the larva ceases to feed and enters the prepupal stage. On emergence of the adult there is a sharp drop in weight amounting to about one-sixth the maximum larval weight.

The water content of a newly laid egg is about 68 per cent. This increases to 83 per cent at time of hatching. Newly hatched larvae contain about 85 per cent water, but after feeding begins this increases to about 90 per cent. A slight fall in percentage of water occurs when the larva ceases to feed, but increases again in the

prepupal and pupal stages until, in the late pupa, it reaches a high of 93 per cent. The fall in per cent water is gradual in newly transformed adults and several months may be required for it to reach the level of 67 per cent characteristic of old adults.

The gradual change in water content of the new adults is correlated with the slow increase in dry weight, slow sclerotization of the integument, and with changes in color from red to black.

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THE QUESTION OF THE SYNTHESIS OF THIAMIN BY THE CILIATE, GLAUCOMA PIRIFORMIS

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Even in the absence of conclusive evidence it has sometimes been assumed that thiamin is synthesized by the plant-like flagellates, either from relatively simple substances or from the thiazole and pyrimidine components of this vitamin. Specific evidence that *Chilomonas paramecium* synthesizes the diphosphopyridine nucleotide (Hutchens, Jandorf and Hastings, 1941) has furnished a real basis for the assumption that Phytomastigophora can synthesize vitamins of the "B complex." Among the higher Protozoa, on the other hand, synthesis of thiamin has been reported so far only for *Acanthamoeba castellanii* (Lwoff, 1938) and for two ciliates, *Tetrahymena geleii* and *T. vorax* (Kidder and Dewey, 1942). In each of these three cases the conclusion is based upon a premise which seems open to question—an unproven assumption that the basal medium was free from thiamin. Other investigations seem to have demonstrated that certain ciliates—*Colpidium striatum* (Elliott, 1937, 1939), *Glaucoma piriformis* (Lwoff and Lwoff, 1937, 1938; Hall, 1944), *Colpidium campylum* (Hall, 1940, 1942), *Colpoda duodenaria* (Tatum, Garnjobst and Taylor, 1942)—require thiamin from exogenous sources. Such a requirement parallels that of the higher animals and, with evidence from other sources, suggests that the higher Protozoa are more specialized than the plant-like flagellates with respect to vitamin requirements. Consequently, the possible synthesis of thiamin by ciliates is a question of some interest.

In attempts to develop a suitable medium for the study of vitamin requirements in *Glaucoma piriformis,* the writers have tried various protein preparations including a "vitamin-free casein" described as free from water-soluble and fat-soluble vitamins on the basis of tests with white rats. This type of casein was used previously by Dewey (1941) and by Kidder and Dewey (1942) in their work with ciliates. Kidder and Dewey concluded that *Tetrahymena geleii* and *T. vorax,* grown in a solution of this casein, could synthesize thiamin in the presence of a certain supplement ("Factor S"). Our experience with this "vitamin-free casein" forced us to consider the possible synthesis of thiamin by *Glaucoma piriformis.*

Material and Methods

The strain of *Glaucoma piriformis* was the one used by Hall and Shottenfeld (1941) and by Hall (1941, 1944). The ciliates were grown in the following basal media:

(1) Casein medium:

"Vitamin-free casein" (Casein-Harris)	10.0 gm.
Distilled water	1.0 liter

(2) Salted casein medium:

"Vitamin-free casein" (Casein-Harris)	10.0 gm.
$MgSO_4 \cdot 7H_2O$	0.2 gm.
K_2HPO_4	0.2 gm.
$CaCl_2 \cdot 2H_2O$	0.1 gm.
$FeCl_3 \cdot 6H_2O$	0.0025 gm.
Distilled water	1.0 liter

To this solution were added, each in a concentration of 1×10^{-7} gm. per cc., $MnCl_2 \cdot 4H_2O$ and $ZnCl_2$.

(3) Dethiaminized casein medium: A 2 per cent solution of Casein-Harris at a pH above 9.6 was heated in the autoclave for an hour at 122° C. According to previous experience (Elliott, 1939; Hall, 1942) such treatment should reduce the thiamin concentration below that required for growth of the ciliates. After treatment, the casein solution was diluted with salt solution and adjusted to pH 6.9 so as to produce a dethiaminized medium, homologous with the salted casein medium just described.

Each medium, with or without an added growth-factor as desired, was transferred to 16 mm. Pyrex culture tubes plugged with bleached non-absorbent cotton (Johnson and Johnson), and then sterilized in the autoclave at 122° C. for 20 minutes. Distilled water was redistilled in Pyrex glass before use. All inorganic salts were "analyzed reagent quality" (Mallinckrodt). Thiamin hydrochloride (Merck's Betabion), 2-methyl-5-ethoxymethyl-6-amino-pyrimidine and 4-methyl-5-B-hydroxyethyl-thiazole were tested in the basal media as described below. The preparations of thiazole and pyrimidine were obtained through the kindness of Dr. D. F. Robertson of Merck and Company.

In tracing growth of populations and growth in serial transfers, cultures were killed by heat (75–80° C.) before counting. Otherwise, our method of counting was essentially the same as that described previously (Hall, Johnson and Loefer, 1935).

Growth in Casein and Salted Casein Media

These two basal media, with and without added thiamin, were used for serial transfers. Thiamin (1×10^{-6} gm. per cc.) was added to a portion of each medium; the remaining portions served as controls. For transfers 1–3 each medium was adjusted to pH 6.7 before sterilization. At this pH solution of the casein was incomplete and the residue was discarded by decanting. Somewhat better results were obtained in transfer 4 by adjusting the medium to pH 6.9. In subsequent transfers the preliminary pH has been varied from 6.9 to 7.2. In transfer 1, all cultures were inoculated from a stock flask culture of *G. piriformis* in casein-peptone medium, and then incubated at 24 ± 0.2° C. Transfers 2, 3 and 4 were inoculated serially from the preceding transfers after 10, 14 and 14 days, respectively; subsequent transfers, after 1–4 weeks of incubation.

Growth of populations in transfers 1–4 is traced in Figure 1; in addition, transfer 11 of the strain in salted casein medium is described in Figure 3. In transfer 1, all the tubes contained a significant quantity of casein-peptone (1 : 2,000) from the stock culture; in the fourth transfer, serial dilution alone would have reduced the

peptone to not more than 1 : 2,000,000. After the first transfer, *G. piriformis* failed to grow in casein medium, either with or without added thiamin. In the salted media, however, growth occurred in serial transfers. The original strain without added thiamin has been maintained for 14 months (28 transfers), with the observed densities averaging a little more than 40,000 ciliates per cc. A second strain, started more recently from a stock in casein-peptone medium, has been carried for six months (14 transfers). Two subinoculations from the original salted-casein strain and two more direct inoculations from peptone stock cultures have failed to establish a strain in the unsalted casein medium. Accordingly, it

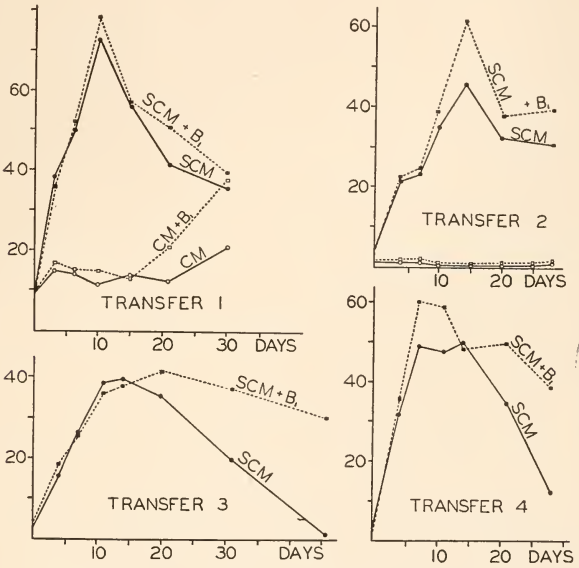

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FIGURE 1. Growth of *Glaucoma piriformis* in casein medium (CM) and in salted casein medium (SCM); B_1, thiamin (1×10^{-6} gm. per cc.). Ordinate, thousands of ciliates per cubic centimeter. Populations are traced in point-to-point curves.

seems that Casein-Harris and a mixture of certain salts constitute a satisfactory culture medium for *G. piriformis,* whereas a one per cent solution of this casein without added salts is inadequate for growth. The mineral requirements of *G. piriformis* are still being investigated and will be considered more specifically elsewhere.

These series were planned to determine the effects of added thiamin, since this vitamin had increased population yield in certain other media (Hall and Shottenfeld, 1941; Hall, 1944) and was known to be essential for growth of *Glaucoma piriformis* (Lwoff and Lwoff, 1938) and certain other ciliates (Elliott, 1937, 1939;

Hall, 1940, 1942; Tatum, Garnjobst and Taylor, 1942). Somewhat surprisingly, added thiamin exerted little influence on maximal density, in distinct contrast to the effects of this vitamin when added to dethiaminized media (Hall, 1942) and depleted media (Hall, 1944). In spite of the comparatively slight effect on maximal density, thiamin did decrease the rate of death following the phase of maximal density—a decrease such as that observed under similar conditions in other media (Hall and Shottenfeld, 1941; Hall, 1944) known to contain thiamin, and likewise in cultures of *Tetrahymena geleii* (Johnson and Baker, 1943). Accordingly, on the basis of previous experience, the effect of thiamin in salted casein medium was just what would be expected in a medium already containing enough of this vitamin

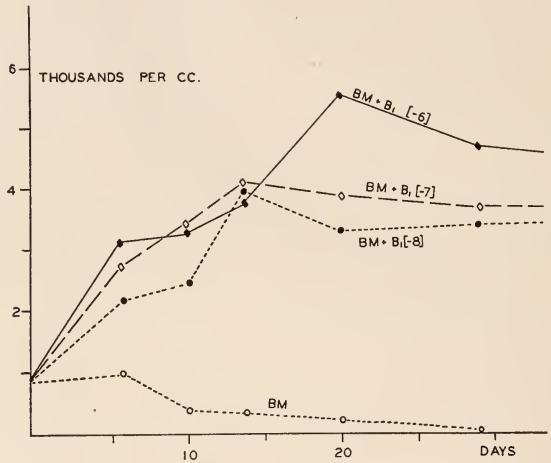

FIGURE 2. Growth of *G. piriformis* in dethiaminized casein medium. BM, dethiaminized medium; B_1 (—6), B_1 (—7) and B_1 (—8), thiamin in concentrations of 1×10^{-6}, 1×10^{-7} and 1×10^{-8} gm. per cc., respectively. The last point on each curve, at 70 days of incubation, is not included in the figure. All four series were started from one flask of dethiaminized medium seeded from a stock culture in salted casein medium (the original strain at transfer 9). The initial count was 850 ciliates per cc.; the initial pH, 6.9.

to support growth of *G. piriformis* to approximately maximal density, as defined by factors other than the supply of thiamin. In other words, there is no evidence that the available concentration of thiamin was a factor appreciably limiting the density attainable in salted casein medium. Hence, it seemed likely that Casein-Harris contains enough thiamin to support moderate growth of *G. piriformis* and that growth in the plain casein medium was limited by a mineral deficiency.

On the other hand, synthesis of thiamin has been attributed to *Tetrahymena geleii* and *T. vorax* grown in a medium (1.0 per cent solution of Casein-Harris with certain supplements) said to be "free of thiamin or its intermediates" (Kidder and Dewey, 1942). If Casein-Harris contains no thiamin, then *G. piriformis* either does not require thiamin or else synthesizes the vitamin from substances in our

salted casein medium. Although the observed effect of added thiamin would seem rather strange if *G. piriformis* is able to synthesize all the thiamin it needs, the possible thiamin requirements of this ciliate were tested with dethiaminized medium.

Growth in Dethiaminized Medium

The effects of adding thiamin to dethiaminized medium were determined by the serial transfer technique and by tracing populations in the first transfer. The method of inoculation was such that relatively fresh casein from the stock culture was reduced to less than 1 : 7,500 in the cultures of transfer 1. A control series was started in the basal medium, while three other series received thiamin in the indicated concentrations (Fig. 2).

In the first transfer (Fig. 2) no growth occurred in the basal medium and no active ciliates were seen in this medium after four weeks of incubation. With added thiamin, however, the populations reached 4,000–5,500, approximately, the highest density being obtained with the highest concentration of thiamin. At the end of 10 weeks cultures with thiamin still contained 2,500–3,500 ciliates per cc.

Subsequently, the ciliates have been grown in serial transfers (Series II, Table I) for nine months (15 transfers) in dethiaminized medium with added thiamin (1×10^{-6} gm. per cc.). The series with lower concentrations of thiamin were discontinued after the first transfer. The strain in the basal medium failed to survive beyond the fourth transfer. In a later attempt to establish a subsidiary strain in dethiaminized medium, series III (Table I) was started from series II at transfer 4. In this case also, the ciliates failed to survive after the fourth transfer.

Table I

Growth of Glaucoma piriformis *in dethiaminized casein medium*

Transfer	Series I, basal medium	Series II, basal + thiamin	Series III, basal medium
1	++	++++	
2	+	+++	
3	+	+++	
4	±	+++	
5	0	+++	+++
6	0	+++	+
7		+++	±
8		+++	±
9		+++	0
10		+++	0
11		+++	
12		+++	
13		+++	
14		+++	
15		+++	

++++, more than 2,500 ciliates per cc.
+++, 1,100–2,000 ciliates per cc.
++, 500–1,000 ciliates per cc.
+, less than 500 ciliates per cc.
±, very few ciliates, viability test positive.
0, no ciliates seen, viability test negative.

These observations indicate that *G. piriformis* requires thiamin for growth and are in agreement with the findings of Lwoff and Lwoff (1938) for this species. The failure of dethiaminized casein medium to support growth of *G. piriformis* presumably indicates that thiamin, present in adequate concentration in Casein-Harris, was largely destroyed by heating the casein in alkaline solution. Such an interpretation of similar results forms the basis for conclusions that various Trypanosomidae and certain ciliates—*Colpidium striatum* (Elliott, 1937, 1939) and *Glaucoma piriformis* (Lwoff and Lwoff, 1938)—require thiamin for growth.

The continued growth of *G. piriformis* in salted casein medium might be explained on the basis that synthesis of thiamin is catalyzed by one or more of the added salts. On the other hand, since *G. piriformis* did not grow in unsalted casein solution with added thiamin, it seems likely that the casein solution is deficient in minerals essential to growth and hence will not support growth even when thiamin is added. However, it is conceivable that any salts, which might be essential as catalysts in biosynthesis of thiamin, could be required also for growth of *G. piriformis* and for utilization of thiamin by this ciliate. Hence, in our experiments with dethiaminized medium the usual salt mixture was added *after* heating the casein in alkaline solution. In this way, unfavorable changes were limited to constituents of the casein solution. It will be noted (Fig. 2; also, Table I) that the addition of thiamin to dethiaminized medium did not restore the population to a normal level, although insuring growth in serial transfers. This limited effect of thiamin must be attributed to some change in the casein itself during dethiaminization, and not to a deficiency in any salts which may be involved in utilization or synthesis of thiamin.

A clue to the nature of such additional changes in dethiaminized casein was obtained by the addition of both riboflavin (1×10^{-7} gm. per cc.) and thiamin (1×10^{-6}) to dethiaminized medium. The density of population was raised to a level approaching that in fresh salted casein medium—in serial transfers, the density has ranged from approximately 20,000 to 35,000 per cc. after different periods of incubation. This relation of riboflavin to growth of *G. piriformis* has been observed also in depleted media and will be discussed elsewhere (Hall, 1944), but it may be pointed out that the present results suggest the presence of both riboflavin and thiamin in Casein-Harris.

Effects of Thiazole and Pyrimidine

The results obtained with dethiaminized medium indicated that *G. piriformis* requires thiamin for growth and that an adequate supply of riboflavin also is needed for normal population yields. If Casein-Harris actually contains neither thiamin nor its components, as implied by Kidder and Dewey (1942), the ability of *G. piriformis* to synthesize thiamin would have to be fairly extensive in order to produce the growth curves described in Figures 1 and 3. Such a ciliate, if it is to synthesize thiamin, should be able to utilize the thiazole and pyrimidine components in biosynthesis, since this appears to be true for microorganisms known to synthesize this vitamin. Accordingly, addition of thiazole and pyrimidine to salted casein medium might be expected to produce a beneficial effect comparable to that of added thiamin.

In considering this possibility, the growth of *G. piriformis* in salted casein

medium was compared with that in media containing: (a) the thiazole and pyrimidine components of thiamin; and (b) thiamin. The results (Fig. 3) show no beneficial effects of added thiazole and pyrimidine, since the population receiving these supplements did not differ significantly from that in the basal medium during 52 days of incubation. Added thiamin, on the other hand, produced the usual lower rate of death after the phase of maximal density. We are forced to conclude that *G. piriformis* did not synthesize thiamin from thiazole and pyrimidine in any significant quantity, and that the relatively rapid death of the ciliates in the basal medium and with the added components of thiamin was the result of a thiamin deficiency.

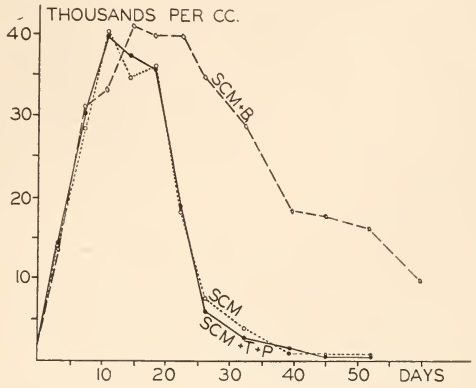

FIGURE 3. Growth of *G. piriformis* in salted casein medium. SCM, salted casein medium; B_1, thiamin (1×10^{-6} gm. per cc.); P, 2-methyl-5-ethoxymethyl-6-amino-pyrimidine (1×10^{-6} gm. per cc.); T, 4-methyl-5-B-hydroxyethyl-thiazole (1×10^{-6} gm. per cc.). All three series were inoculated from a flask of the basal medium seeded from a stock in salted casein medium (the original strain at transfer 10). The initial count was 173 ciliates per cc.; the pH, 6.8.

By means of the serial-transfer technique, the effects of added thiazole and pyrimidine were determined also in dethiaminized casein medium. Three series were started—one with the basal medium, a second with added thiazole and pyrimidine (concentrations as in Fig. 3), and the last with added thiamin (1×10^{-6} gm. per cc.). The initial pH in transfer 1 was 6.9, and all three series were inoculated from transfer 10 of series II (Table I). The series with added thiamin was discontinued after the sixth transfer, growth having approximated very closely that in corresponding transfers of series II (Table I). In the other two series, very little growth occurred in the fourth transfer, no normal ciliates were seen in the fifth, and viability tests (casein-peptone medium) of the sixth transfer cultures were negative. No beneficial effects of added thiazole and pyrimidine were detected, and these substances obviously could not be substituted for thiamin in dethiaminized casein medium.

Discussion

The results obtained with *Glaucoma piriformis* afford no basis for concluding that this ciliate synthesizes thiamin under the conditions of our experiments. Yet our basic findings for this species coincide with those reported for *Tetrahymena geleii* and *T. vorax* (Kidder and Dewey, 1942). All three of these ciliates failed to grow in a one per cent solution of Casein-Harris. Furthermore, the addition of thiamin did not enable the organisms to grow in serial transfers. Such a casein solution obviously is an inadequate medium for these ciliates, and the failure of added thiamin to correct the deficiency would seem to demonstrate that this casein preparation lacks some other substance or substances essential to growth.

Kidder and Dewey (1942, table 6) found that a combination of casein solution and a "heat and alkali treated water extract of alfalfa meal" supported growth of their ciliates. These results form the basis for their conclusion: "Two of the ciliates (*Tetrahymena geleii* and *T. vorax*) were found to be able to synthesize thiamin if a substance from leaves, Factor S, was added to a medium free of thiamin or its intermediates." Such an assumption does not explain the failure of *T. geleii* and *T. vorax* to grow in casein solution with added thiamin. In other words, if growth is possible when an added catalyst enables the ciliates to synthesize thiamin from an otherwise satisfactory medium, then growth should occur also when exogenous thiamin is supplied.

The writers have shown that the addition of certain salts to casein solution (Casein-Harris, one per cent) insures growth of *G. piriformis* in serial transfers. In view of these results, it might be assumed that synthesis of thiamin is catalyzed by certain of the added salts. However, there would remain the facts that *G. piriformis* did not grow in simple casein solution with added thiamin, and that these same salts did not enable *G. piriformis* to synthesize thiamin in dethiaminized casein medium. Consequently, it seems entirely probable that the casein solution is deficient in essential salts and without such supplements will not support growth even when thiamin is added.

Populations of *G. piriformis* in dethiaminized casein medium and in depleted media (Hall, 1944)—media which are apparently unsuitable for synthesis of thiamin by this ciliate—have been found to benefit greatly from added thiamin. In fact, there has been no growth in certain instances without added thiamin. Thus it is obvious that growth of *G. piriformis* depends upon a readily available supply of thiamin. If it is true that our salted casein medium contains no thiamin, then added thiamin should exert an immediate influence on growth of populations. Instead, the densities in the basal medium and in that containing thiamin as a supplement were not significantly different until after the populations reached maximal density (Figs. 1, 3). Such results would not be expected if Casein-Harris were actually free from thiamin.

The conclusion of Kidder and Dewey, that *T. geleii* and *T. vorax* synthesize thiamin when supplied with "Factor S," is based primarily upon the assumption that Casein-Harris contains no thiamin. It must be pointed out, however, that this casein is biologically standardized with the white rat as the test organism and *on that basis* is described as vitamin-free. Kidder and Dewey have presented no additional evidence. Actually, Casein-Harris might well contain enough thiamin for moderate growth of *G. piriformis,* since nicotinic acid (3.6 micrograms per gram)

has been demonstrated in a "vitamin-free casein" (Noll and Jensen, 1941). Furthermore, it has been reported (Halliday and Duel, 1941) that about 40 per cent of the thiamin in milk is in non-dialyzable form, probably combined with proteins in a complex which can be broken down by such a proteinase as papain, a type of enzyme produced by *G. piriformis* (Lawrie, 1937). Since Casein-Harris is washed by reprecipitation and extraction with dilute acids, the treatment might leave a little residual thiamin—enough to support growth of *G. piriformis* as observed in serial transfers.

Accordingly, the most probable interpretation of our data involves the following conclusions: (1) Casein-Harris does not contain an adequate supply of certain minerals essential to growth of *G. piriformis*. (2) The effects of thiamin, added to our salted casein medium, are similar to those produced in other media known to contain thiamin—there was little if any increase in population, although the rate of death following the phase of maximal density was lower than in the control medium. (3) The effects of thiamin, added to salted casein medium, are decidedly different from those produced in dethiaminized and depleted media, in which the density of population is increased promptly. (4) Casein-Harris apparently contains enough thiamin to support growth of *G. piriformis* in serial transfers. (5) The failure of added thiazole and pyrimidine to influence growth, either in salted casein medium or in dethiaminized casein medium, affords no evidence that *G. piriformis* utilizes these components in the synthesis of thiamin. (6) Our findings supply no evidence that *G. piriformis* can synthesize thiamin in casein medium, in salted casein medium, or in dethiaminized casein medium.

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THE MECHANISM OF EXTENSION IN THE LEGS OF SPIDERS[1]

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Introduction

Prior to 1909 morphologists studying the leg musculature of spiders unanimously considered movement in each joint to be controlled by antagonistic muscles. The femoro-patellar and tibio-metatarsal joints (the two most important flexor-extensor joints in the leg), however, are not supplied with such paired muscles, since there are no extensor muscles (Petrunkevitch, 1909). The alacrity of extension in these joints, therefore, presents the singular problem of accounting for these "muscle-less" extensor movements.

The assumption has been made by Petrunkevitch (1909, 1916, personal communication 1941) that "the function of extension has been taken over entirely by the elastic interarticular membrane." Except for this single suggestion by Petrunkevitch, no mechanism has been forwarded to account for these forceful and rapid extensor movements. It has seemed worthwhile, therefore, to investigate this problem.

The spiders used in the present study have included the tarantulas, *Dugesiella californica, Delopelma hentzi*, and *Aphonopelma cryptethus* representing the suborder Mygalomorphae, and *Agelena naevia* (the grass spider), *Miranda aurantia* (the golden garden spider), *Latrodectus mactans* (the black widow), and *Aranea cavicata* (large orb-weaver) representing the suborder Dipneumonomorphae. All of the specimens were collected in or near Pasadena, California.

With respect to the morphological and functional relationships studied in the present investigation, no significant differences between the species were found.

Results and Discussion

A. *The possibility of extension by muscular contraction*

That muscular contraction is not the extensor mechanism is obvious. In agreement with Petrunkevitch it has not been possible to find any trace of extensor muscles (see below) in the two joints under consideration. Furthermore, the axis in each of these joints is located dorsally in such a way as to preclude attachment of extensor muscles. At these joints the appendages are flattened over the dorsum in the articular region, and are joined by very narrow dorsal interarticular membranes. During flexion or extension these membranes serve as simple hinges. With this extreme dorsal location of the axis there is no conceivable way in which

[1] This investigation formed a portion of a thesis submitted to the Faculty of the Graduate School of California Institute of Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in June, 1943.

internal muscular attachments could be made whereby muscular contraction would produce extension of the joint, and it is certain that no tendons pass externally over the hinge. Therefore, the theory of muscular extension must be discarded.

B. *The possibility of extension by the interarticular membrane*

There are two interarticular membranes (Fig. 1 dm, vm), one dorsal, the other ventral. Of these the dorsal one is very short. Petrunkevitch (1909) claimed for this dorsal membrane the un-assisted function of extension, but examination of his paper has not given the experimental basis upon which he based this conclusion.

Were the elasticity of this membrane the mechanism involved in extension it would seem imperative that the spider assume a position of complete extension in death. This is not the customary position taken; spiders nearly always die with their legs completely and permanently flexed. In fact, so characteristic is the flexed position in death that when startled a number of spiders assume the position as a protective reflex (Robertson, 1904).

It might be reasoned that this position of extreme flexion is due to an irreversible contracture in the muscles. That such a mechanism is not responsible for this flexed position was shown by the following experiment: A leg was removed at the trochanter and the chitin covering the ventral half of the femur was removed, due care being taken to leave both the ventral and dorsal interarticular membranes intact. The muscles in the femur were transected as distally as possible, and those in the patella were loosened at their origin. The same procedure was carried out in the tibio-metatarsal joint, and in neither case did extension occur. In fact, the joint remained in precisely that position in which it was manually placed. The legs from spiders of a number of different genera were used, and in all instances these results were observed. It is obvious, therefore, that the membranes themselves have little if any elasticity. This would certainly appear to render untenable any theory basing either extension or flexion on an inherent elasticity of the interarticular membranes.

C. *The effects of electrical stimulation*

In the following experiments the leg was removed at the trochanter and was anchored at the femur to the stage of a binocular dissecting microscope with plasticine. In most instances the tip of the tarsus was attached to a very lightly weighted heart lever by a length of silk thread, and the movements were recorded on a smoked kymograph drum. Stimulation was produced with micromanipulated platinum electrodes using a DuBois-Reymond inductorium.

Various ways of stimulating were used in an effort to produce extensor movements. The nerve was prepared in the femur and was stimulated either in its entirety or in bundles after splitting the nerve with a fine needle. In some instances the tendons of the flexor muscles were cut prior to stimulation of the nerve. In all instances, however, stimulation with currents of ordinary intensity resulted only in flexor contractions if any response was obtained. This supports the finding that no extensor muscles are present.

The functions of the respective muscles in the segments of the leg distal to and including the femur have been investigated by selectively stimulating each of the

muscles. Most of these studies were made on the walking legs of tarantulas, although some observations have been made on the legs of *Agelena naevia, Latrodectus mactans,* and *Miranda aurantia.* No differences between the species were found.

The leg was opened with a pair of small scissors and the chitin from the side opposite the muscle to be examined was removed with a pair of fine forceps. Small platinum electrodes mounted in Zeiss micromanipulators were then applied to the muscle with the aid of a dissecting microscope. Contraction of the muscle was ob-

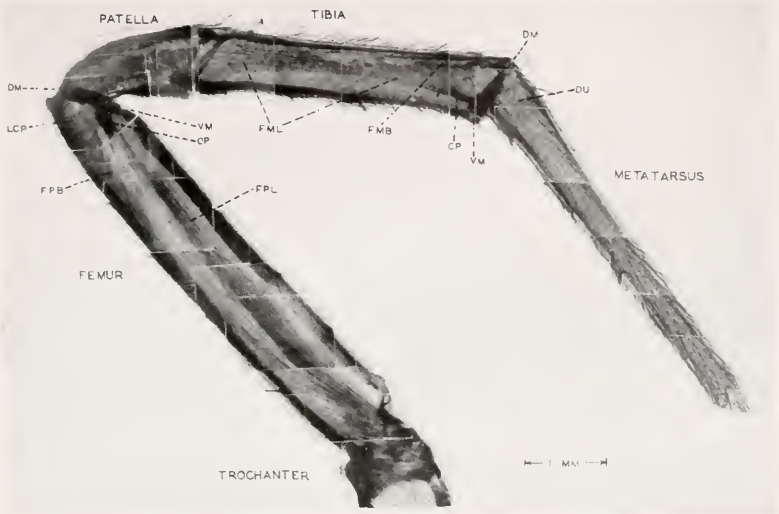

FIGURE 1. Composite photomicrograph of the mid-sagittal section of a walking leg of *Miranda aurantia.* CP = chitinous plate, DM = dorsal interarticular membrane, DU = depressor unguium muscle, FMB = flexor metatarsi bilobatus, FML = flexor metatarsi longus, FPB = flexor patellae bilobatus, FPL = flexor patellae longus, LCP = levator of chitinous plate, VM = ventral interarticular membrane.

served under the microscope to prove the selectivity of stimulation and movements of the parts of the leg were observed visually.

The muscles of the femur showed the following functions: The *flexor patellae bilobatus* (Fig. 1 fpb) produced strong flexion of the femoro-patellar joint. As Petrunkevitch (1909) and Brown (1939) reported, this bitendonous muscle lies laterally and dorsally in the femur, arising from the dorsal femoral surface and inserting directly on the proximal lip of the patella. The *flexor patellae longus* (Fig. 1 fpl), arising from the ventro-lateral lip of the trochanter and from the ventral proximal portion of the femur, extends ventrally and medially the full length of the femur, inserting, not on the proximal lip of the patella as has been described

(Petrunkevitch, 1909), but on the proximal margin of a chitinous horseshoe-shaped plate to whose distal margin are attached the proximal and distal portions of the ventral interarticular membrane (Fig. 3 cp). Stimulation of either of the bellies of this muscle likewise resulted in a flexor movement, although of considerably less intensity than that of the *flexor patellae bilobatus*.

An homologous picture was found in the tibia; the strongest flexor movement was obtained from stimulation of the *flexor metatarsi bilobatus* (Fig. 1 fmb), while the movements resulting from stimulation of the *flexor metatarsi longus* (Fig. 1 fml), although also flexor in nature, were much weaker than those of the former muscle.

Stimulation of the *depressor unguium* (Fig. 1 du) in the tibia, of the *levator unguium* in the metatarsus, and of the two main muscles in the patella gave the expected results, i.e., depression and elevation of the claw and the respective lateral movements of the tibia.

It has thus been possible to confirm most of the morphological findings of Petrunkevitch (1909) and Brown (1939), and to establish at the same time a clear functional proof of the specific activities of most of the muscles in the spider leg.

It was observed that "closed-leg" preparations, i.e., those in which the electrodes were inserted through the chitin of the femur, gave extensor movements of the two joints under consideration when stimulated very strongly for several seconds (coil distance = 3 to 1½ cm.). Upon removing the tip of the tarsus, however, such stimulation resulted in a much less pronounced extensor movement and was accompanied by the appearance of a growing droplet of blood at the severed end. Sealing the tip with plasticine resulted in a restoration of extensor movements of the original height. This stimulation was of such intensity that bubbles of hydrogen and oxygen appeared on the electrodes, and it is likely that the accumulation of gases was instrumental in producing the extensor movements.

D. *The possibility of an hydraulic extensor mechanism*

If the liberation of gases by electrolysis is sufficient to so increase the internal pressure of the leg that a straightening of the appendage occurs, it would seem quite feasible to consider that increases in the fluid pressure within the leg could result in extension. That such an increase does result in an extensor movement can be shown by gently squeezing an isolated leg. Extension occurs at the joints with a ballooning of the ventral interarticular membrane. Gaubert (1892) reported the phenomenon and considered it indicative of an extensor mechanism accessory to the "extensor muscle" which he firmly believed to exist.

Injection of fluid with a hypodermic syringe into any part of the leg, whether isolated or on the living or dead animal, is accompanied by extension of all parts distal and proximal to the point of injection. Injection into the abdomen or cephalothorax also produces extension of the appendages. The pressure necessary for producing this effect is small, a very light touch on the plunger of the syringe being sufficient to produce extension. Petrunkevitch (1910), although not mentioning the possibility of an hydraulic extensor mechanism, observed the above phenomenon, and used it as an indication of the completeness of injection while studying the circulatory system of spiders. He said, "Die Injektion musz so lange fortgesetzt werden, bis alle Beine straff auseinandergespreizt bleiben und die

Stacheln an ihnen nahezu senkrecht abstehen." Ten Cate (1931) showed that in Celaenia cutting off the abdomen resulted in stretching of the legs, but once the abdomen had been removed, the animal fell down as in death, i.e., the legs were flexed and no further extensor movements occurred. It would appear obvious that the constriction during the cutting process would momentarily increase the pressure giving rise to the extensor response.

That such hydraulic extension can be brought about mechanically does not give sufficient grounds, however, for assuming that the same mechanism is involved in the normal extensor movements of the live animal. The following experiments were therefore undertaken in an effort to see whether or not any similar pressure changes normally occur.

In the intact tarantula cutting off the tip of a leg produces a condition in which that leg is not extended upon stimulation of the animal in a manner which normally results in extension of the appendages, i.e., holding the animal in an inverted position. Sealing the tip restores the extensor function. The same effect has been obtained by opening the chitin of the femur in the intact spider. In such an animal pressure applied distal to the opening results in extension of the two joints, but pressure applied proximal to the opening results only in a marked effusion of fluid from the hole. Moreover, so long as the hole is open, the animal moves around with the operated leg in a flexed position, whereas the unoperated legs show normal extensor activity. The same result was obtained in *Agelena naevia*.

Internal fluid pressure. Spiders always appear to be maintaining a high internal pressure within their bodies and appendages, as is seen by the fact that even a small perforation of the chitin of any living spider results in a great loss of blood whether the hole be in the abdomen, cephalothorax or appendage. In order to investigate the effect of lowering this internal fluid pressure, a number of tarantulas were dehydrated by keeping them for several weeks without water. They showed, prior to death, a condition in which walking was not accompanied by extension of either the femoro-patellar or tibio-metatarsal joints, and the spiders walked with their legs in a more or less markedly flexed position. In such an animal (tarantula) injection of 2 ml. of a salt solution [2] was accompanied by a period in which the two above-mentioned joints were extended as readily as in the normal animal. It should be recalled, however, that any perforation is accompanied by a considerable loss of blood, and upon removal of the injection needle from the abdomen, the animal bled profusely and in a short time again lost the ability to extend the legs. A second injection of physiological solution restored the normal condition, and when the hole was closed with collodion the animal ran about the cage in a more normal way, making use of its extensor mechanism.

The blood volume of the spider has also been lowered experimentally by withdrawing an appreciable amount of blood, using a small hypodermic needle, whereupon extension became markedly slower and less effective. Replacing the withdrawn blood with physiological solution gave a return of quick extensor movements of normal magnitude.

[2] Frog Ringers and several other salt solutions were unsatisfactory. Since these solutions had a molar concentration considerably lower than that of the physiological solution for crayfish described by van Harreveld (1936), the latter fluid was tried. It proved to be quite satisfactory, the animal remaining alive for somewhat longer than one hour following injection.

It has been pointed out that spiders characteristically die with their legs in a strongly flexed position. An interesting exception to this rule was observed in the case of a male tarantula which fell into its dish of drinking water and drowned. In this animal the legs were almost fully extended. Furthermore, the slightest pressure applied to the abdomen was sufficient not only to extend the legs maximally, but to raise the spines as well. It is evident that the spider in this case had imbibed water to such an extent that an abnormally high fluid pressure was present.

From the foregoing experiments the conclusion can be drawn that the internal fluid pressure of the spider must be kept high if maximum extensor activity is to be maintained.

Cardiac pulsations in the leg. Plateau (1887) and Gaubert (1892) observed that when spiders are suspended so that one or more of the legs is without a point of rest, those legs successively extend and flex through a small arc synchronously with the systolic and diastolic phases of the heart beat. Willem and Bastert (1918) and Willem (1920) made use of this movement of the tarsi to record changes in the cardiac rhythm when the spider was stimulated by various means, e.g., shining a bright light in the eyes of the animal. They showed that these flexor-extensor movements of the tarsi were more rapid during systole (extension) than during diastole (flexion), and that these movements could thus be considered to be a true picture of the sequences of cardiac pulsation. It has been possible to confirm this synchronization of rhythmic leg movements with the heart beat in the crab-spider, *Misumena alcatoria,* in which the movement of the dorsal chitin of the abdomen with each heart beat is conspicuous. Only the femoro-patellar and tibio-metatarsal joints showed the above phenomenon, and the ventral thin-walled sac at each of these joints showed ballooning with the extensor phase of each movement.

The experimental evidence which has been presented indicates that extension in spiders is intimately associated with the internal hydraulic pressure of the leg, which is in turn associated with the blood volume within the appendage. It also seems likely, although not certain, that the claws are spread apart by an increase of hydrostatic pressure. They are pulled together, however, by muscular action, and elevated and depressed by contraction of the appropriate muscles. For the complex movements of the male copulatory apparatus Osterloh (1922) has presented evidence that an hydraulic mechanism is present.

The details of the hydraulic mechanism are not obvious. An increase in the internal fluid pressure could be obtained in several ways. (1) The venous return pathway could be partially blocked, thereby permitting a greater volume of blood to accumulate in the appendage. Some mechanism of this sort must be present, or it becomes difficult to account for the fact that fluid is not forced out of the proximal end of an isolated leg upon compression of the femur. (2) The arterial flow of blood into the leg could be diverted from its normal path so as to produce an effective extensor thrust by ballooning the ventral interarticular membranes. A combination of these mechanisms may be involved.

There are, however, certain peculiarities of structure which have been observed in the two joints in which this type of extensor control is found. These should be considered as to possible roles in extension.

Structural peculiarities. From the ventral tibio-metatarsal and femoro-patellar interarticular membranes arise chitinous plates (one in each joint) which extend

proximally for a short distance. These plates are flattened dorso-ventrally and are bowed proximad. The plate in the femoropatellar joint was described by Gaubert (1892) but its presence has been consistently ignored by subsequent investigators, and the homologous structure in the tibio-metatarsal joint has apparently never been reported. From the proximal margin of these plates arise the tendonous strands whereby the *flexor metatarsi longi* and *flexor patellae longi* are respectively inserted. Moreover, a few isolated muscle fibers, arising from the distal dorsal wall of the femur and inserting on the dorsal surface of the chitinous plate (Fig. 2 lcp), seem to be uniformly present in sagittally cut preparations.[3] It was first thought that these fibers might be artifacts resulting from the cutting procedure, but care was taken to make the cut from different directions with respect to

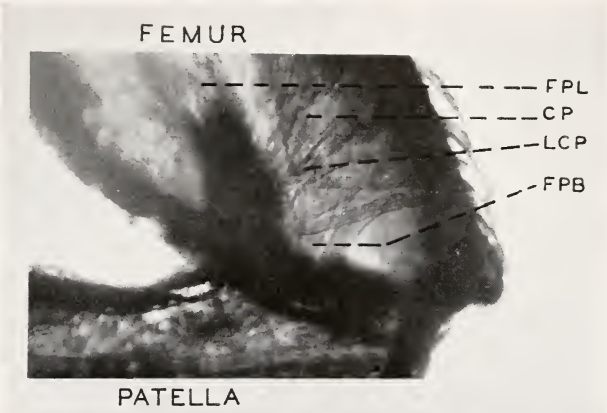

FIGURE 2. Photomicrograph of the femoro-patellar joint of *Miranda aurantia*. (Mid-sagittal section.) CP = chitinous plate, FPB = flexor patellae bilobatus, FPL = flexor patellae longus, LCP = levator of chitinous plate.

the femoro-patellar joint, and in all cases these fibers were found to lie in almost identically the same position on the dorso-ventral median line of the femur. It appears, therefore, that these few fibers form an heretofore undescribed muscle, the function of which is obviously elevation of the chitinous plate. It is probable that this group of fibers has been overlooked because they run in nearly the same direction as the more laterally located distal fibers of the *flexor patellae bilobatus*. Careful examination of Figure 2 will show, however, that these fibers of the large flexor muscle insert on the chitinous lip of the patella, not on the plate. The existence of

[3] The above sagittal preparations were made by first fixing the isolated leg in Petrunkevitch's (1933) copper-tri-nitro-phenol, dehydrating with graded alcohols and xylol, and imbedding in paraffin. The paraffin block was then trimmed under the dissecting microscope with a sharp scalpel until the mid-sagittal plane was reached. The excess paraffin was removed in xylol, the half-leg washed through several fresh xylol baths, and finally mounted in balsam.

an homologous group of fibers elevating the chitinous plate in the tibio-metatarsal joint is still uncertain.

The distribution of the arteries into the muscles and other tissues of the leg has never been adequately described. Causard (1896) figured the pedal arteries as simple uninterrupted tubes, one in each leg, emptying into the cavity of the leg through a single perforation in each segment except the tarsus where the artery ends as an open tube. Petrunkevitch (1910) stated that it is possible to trace the pedal arteries as far as the claw, but said nothing concerning any branching within the leg. Profuse branching of the main pedal artery occurs, however, as can be seen by injecting an animal with a colored latex medium.[4, 5] The branches arborize into the muscles in a manner quite similar to that described by Causard (1896) for the muscles of the cephalothorax (Fig. 3).

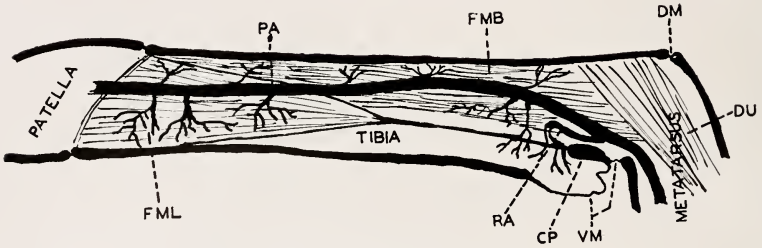

FIGURE 3. Diagram showing the vascularization in the tibia of a spider. CP = chitinous plate, DM = dorsal interarticular membrane, DU = depressor unguium, FMB = flexor metatarsi bilobatus, FML = flexor metatarsi longus, PA = main pedal artery, RA = reflected arterial branch, VM = ventral interarticular membrane.

With respect to a possible structural relation between the distribution of these vessels and the extensor function in the two joints under discussion, two peculiarities should be described. (1) Careful examination of the distribution of the main arterial trunk in the appendage shows that, although it runs somewhat dorsally through the major part of the femur and tibia, on approaching the respective joints, it dips ventrally and passes in close proximity over the chitinous plate. (2) In several preparations of the legs of tarantulas, a rather large branch has been found

[4] In order to obtain any degree of success with injection of the arterial system in the legs of spiders it was found necessary to remove the tips of the tarsi to provide an outlet for the increased pressure created by injection. The method of injection, therefore, was as follows: The spider was killed by placing it in a bottle containing a few drops of chloroform. The diluted alkaline latex solution was sucked up into the injection pipette, the tip of which had been drawn out into a rather fine capillary. The pipette was inserted through the dorsal wall of the abdomen, and the latex medium was forced into the animal with compressed air. Dilute HCl was then injected by hypodermic syringe, forcing the acid into the body and appendages at various places, thus setting the latex. It was found that when the legs were left intact, no injection fluid was able to penetrate the vessels of the appendages, but when the tips of the legs were removed, a most satisfactory injection of even the finest branches was obtained.

[5] The author wishes to express his appreciation to Mr. C. Blair Coursen of the General Biological Supply House, Inc., who kindly made available a sufficient supply of the latex injection medium.

to arise from the main pedal artery just proximal to the plate in the tibio-metatarsal joint. This branch is first reflected in a proximal direction for a short distance, whereupon it dips ventrally, arborizing laterally into the tissues surrounding the membranous sac bounded by the ventral interarticular membrane (Fig. 3 ra).

This distribution suggests that a mechanism for extension might involve the partial closing off of the main arterial flow, thus diverting a greater amount of blood into the region of the membranous sac, ballooning the latter structure and producing extension. Such a mechanism would have the distinct advantage of selectivity, either the tibio-metatarsal or femoro-patellar joint being independently extensible.

In order to examine the role which the plate may play in this function a study of its movement during flexion and extension of the leg has been made. Such motion can be watched through the thin transparent ventral interarticular membrane. The observation has been made that during flexion of the femoro-patellar joint the chitinous plate remains close to the ventral membrane. This is to be expected from the position of the attachments of the *flexor patellae longus* which, lying ventrally, pulls the plate in a horizontal plane. Because of the ballooning of the ventral interarticular membrane by the inflow of blood with each extensor movement it was not possible to determine whether the plate is pulled deeper during extension or whether this only appears to be so because of the ballooning effect. At any rate, one loses sight of the plate during extensor movements. That contraction of the fibers which lift the plate occurs during extension can only be assumed, therefore, and their designation as an auxiliary extensor mechanism must await further investigation.

Summary

The absence of extensor muscles in the legs of spiders presents the problem of accounting for the quick extensor movements characteristic of these animals. The theory that the "elasticity" of the interarticular membrane might be the means whereby this is accomplished has been shown to be untenable.

Experimental evidence has been presented showing that extension is intimately associated with changes in the volume of blood in the spider leg, i.e., with changes in the internal fluid pressure in the leg.

Certain structural peculiarities in the joints have been described, but the evidence is insufficient to permit evaluation of their possible roles in extension.

The author wishes to express his gratitude to Professors C. A. G. Wiersma and A. van Harreveld for their helpful suggestions during this investigation.

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LOCALIZATIONS OF ALKALINE AND ACID PHOSPHATASES IN THE EARLY EMBRYOGENESIS OF THE CHICK [1]

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Since Gomori's publication of a histochemical method for the demonstration of alkaline phosphatase, several studies of the distribution of alkaline phosphatase within tissues have appeared. Gomori himself (1941a) made a survey of the enzyme in a variety of adult mammalian organs; Kabat and Furth (1941) studied its occurrence in normal and tumorous tissues of mammals and chickens; Bourne (1943) investigated its presence in numerous tissues, giving special attention to the intra-cellular localizations; and Horowitz (1942) examined the interrelations of phosphatase and glycogen in fetal heads. Gomori (1941b) employed his somewhat less satisfactory technique for acid phosphatase in a study of acid phosphatase distribution in normal and tumorous tissues, and Wolf, Kabat and Newman (1943), after modifying the original technique to produce more regular results, used it in a study of acid phosphatases in the nervous system.

The present survey of the distribution of phosphatases in the early embryo of the chick was undertaken for two reasons. First it seemed likely that just as the studies cited above have aided in establishing correlations between phosphatase content and function in mature organs, so an embryological study might further elucidate the problem of the functional rôles of phosphatase by establishing correlations between phosphatase content and functional state in developing organs. Second, because of our increasing knowledge of the widespread importance of phosphate transfer in metabolism, it seemed worthwhile to examine the possibility that phosphatases play a part in the proliferative and differentiative processes of the early embryo.

Materials and Methods

These studies are based on White Leghorn embryos taken in stages from the unincubated blastoderm through the eighth day of incubation for all series of observations except those on the long bones of the hind limb, for which nine- and ten-day limbs were also used. At all stages, cold acetone was employed as fixative; after 2 to 24 hours in acetone, depending on size, the embryos were cleared in cedarwood oil and benzene, embedded in a paraffin-beeswax-bayberry wax mixture, and sectioned at 10 or 15 micra. Shrinkage of the cytoplasm occurred in many cells, but for gross histological examination chilled acetone proved an excellent fixative; for the preservation of alkaline as well as acid phosphatase it was superior to 85 per cent alcohol.

The histochemical method for the demonstration of alkaline phosphatase has been repeatedly described (Gomori, 1941a; Kabat and Furth, 1941; Bourne, 1943).

[1] Aided by a grant from the Rockefeller Foundation to Washington University.

The method was employed in these studies exactly as presented by Gomori, except that sodium glycerophosphate containing equal parts of the alpha and beta salts was used, since pure alpha glycerophosphate was not available; the pH was 9.3, and the time of incubation 1½ to 4 hours, for different specimens, at 38° C. Erythrosin was used as counterstain.

In the case of the acid phosphatase, Gomori's (1941b) formula for the incubating solution was altered to compensate for the precipitation of lead beta-glycerophosphate, as follows:

Acetate buffer at pH 4.8	2½	parts
5% lead nitrate	1	part
Distilled water	15	parts
2% Na glycerophosphate	3	parts

The ingredients should be mixed in the order given, and the solution be allowed to stand overnight in the refrigerator, since the precipitate develops slowly. The solution was filtered just before use, at which time any additional reagents were added. The pH is 5.1; the incubation time ranged from 5 to 16 hours, at 38°, since the concentration of acid phosphatase in most embryonic tissues is extremely low. In some cases at each stage 0.01 M $MnSO_4$ was added to the incubating solution in order to produce the stable black-staining precipitate described before (Moog, 1943b); and after it was discovered that ascorbic acid is a strong activator of acid phosphatase, series were also prepared with 0.01 M ascorbic acid added to the incubating solution. This phenomenon will be discussed in a later section.

After incubation the slides were rinsed in distilled water and dilute acetic acid, and stained by 2 minutes' exposure to light yellow ammonium sulfide, which results in the formation of brown or black lead sulfide. After thorough washing under the tap, the slides were dehydrated in the usual manner, counterstained with erythrosin, and mounted.

At all stages controls were made to check on the possible presence of preformed phosphates, by incubating slides in a solution from which glycerophosphate was omitted, and then staining them in the usual way. To a considerable extent also the alkaline and acid series served as controls for each other, since any tissue appearing negative to either enzyme could not contain preformed phosphate.

The stability of alkaline phosphatase in chick embryos has already been remarked on. The acid phosphatase is however troublesome to demonstrate with any regularity, so that material used for the study of this enzyme must be handled with great caution (cf. Moog, 1943a). The use of manganese improves the production of clearly visible precipitates in tissues low in acid phosphatase, but it does not control the considerable variations in activity found among individual embryos. Ascorbic acid, on the other hand, has proved valuable by reason of its ability to increase both the intensity and the uniformity of the normal deposits, especially in fresh preparations. Yet even with ascorbic acid variations continue to appear, and seem to reflect the extreme lability of the enzyme; for example, it will survive only a few days' storage in the refrigerator, even in paraffin, and it is quickly inactivated by temperatures above 60° C. The penetrating power of acetone was not a limiting factor, as in the tissues Wolf, Kabat and Newman (1943) used, for the deepest layers of the liver, even in eight-day embryos, gave more consistent results than some of the more superficial tissues.

Results

I. Alkaline Phosphatase

In the embryonic stages studied, alkaline phosphatase has been found present more generally in nuclei than in cytoplasm. On the basis of the relative concentration of the enzyme in nucleus and cytoplasm, in fact, three types of tissue can be described. (1) Those tissues, or cell-aggregations, in which both nuclei and cytoplasm stain intensely: most tissues show this condition during the first two or three days of development, or in some cases much longer, while in several tissues this condition is achieved in the course of differentiation. (2) Those tissues in which the nuclei are more reactive than the cytoplasm: this distribution is found in some regions very early, before the peak of phosphatase concentration is reached, and again it is found commonly later, when many tissues gradually lose their early reactivity as differentiation proceeds. A decrease in enzyme content always affects the cytoplasm before the nuclei. (3) Those tissues in which neither cytoplasm nor nuclei show marked phosphatase activity: in a few cases this condition obtains throughout the period studied, but more often the relatively phosphatase-free state results from the gradual disappearance of the enzyme from originally reactive cell groups during the course of differentiation. The nuclei are never found to be less reactive than the cytoplasm, nor do they ever become completely negative, although the cytoplasm frequently does so. In the least reactive nuclei, the nucleoli stain heavily and the nuclear membranes lightly. Where the phosphatase concentration is greater, the membranes stain more darkly, the karyoplasm takes on a deepening greyish coloration, and intensely stained masses make their appearance within the membrane; these masses are probably chromosomal detritus, since the chromosomes would be expected to disintegrate during several hours exposure to a solution at pH 9.3.[2] Finally, when the phosphatase concentration is very high, the nuclei stain solid black within a relatively short time. The usual high reactivity of the nuclei makes it sometimes difficult to decide whether a change in phosphatase concentration is real, or merely the apparent result of either lessened crowding of the nuclei or an increase in the amount of cytoplasm per nucleus. To rule out errors due to these factors, it was necessary to make careful comparisons of treated sections with others stained with ordinary nuclear stains.

In the descriptions which follow, the terms "positive" and "active" or "reactive" have been applied to tissues which show more than the minimal nuclear response described above (i.e., no deep stain except in the nucleoli). Those tissues which show only the minimal nuclear response are denoted "negative." An attempt has been made to rate both alkaline and acid phosphatase concentrations on a single scale on which 16 + represents a solid black deposit appearing after 1½ hours' incubation, and 1 + the lightest recognizable deposit appearing after 16 hours' incubation.

The first day

In the unincubated blastoderm both the incipient germ layers and their neighboring yolk cells contain alkaline phosphatase. The smaller, compact yolk granules (white yolk) clustered in the marginal zone are very strongly positive (12 +), the

[2] I am indebted to Dr. Jack Schultz for pointing this out to me.

larger granulated elements (yellow yolk) being much less so. The endoderm and ectoderm are about equally positive (9 +), but their reactivity is much greater in the heavily staining nuclei and in the enclosed yolk granules than in the cytoplasm. At the periphery of the blastodermal area the phosphatase content of the ectoderm wanes.

From the blastoderm sections it appeared that the white yolk is more rich in phosphatase than the yellow, which however seemed to contain a moderate amount of the enzyme also. But smears of yolk taken from regions away from the blastoderm revealed that neither type of yolk contains much phosphatase, although both take on a spurious stain due to the presence of preformed phosphates. The high activity of the white yolk around the embryonic area thus seems rather to be related to proximity to the embryonic tissue, than to be an intrinsic property of white yolk.

As the embryonic body itself is laid down toward the close of the first day, it has at the anterior end a phosphatase content as high as in the preceding tissues, or slightly higher in the case of the ectoderm; but there is a gradual decrease in all layers toward the posterior end. Thus a fairly uniform antero-posterior gradient in intensity of staining can be observed. As before, the reaction also decreases in the superficial ectoderm of the extra-embryonic area.

Two and three days

The brain, and the mesoderm at anterior levels, stain virtually solid black during the second day (Fig. 2). Posterior to the brain the ectoderm and mesoderm are slightly less reactive, and in the entoderm the nuclei stain only moderately, while the cytoplasm is almost negative. At the posterior tip of the body all tissues are still only slightly reactive (Fig. 3). Through the bulk of the body, however, the brain, nerve cord, mesoderm, endothelia, and also the notochord, contain considerable amounts of phosphatase (8 + to 12 +). The red blood cells are negative. The nuclei of the extra-embryonic membranes occasionally show more than

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PLATE I

1, 6—acid phosphatase
2–5, 7, 8—alkaline phosphatase

1. Through primitive groove. *ec*, ectoderm; *m*, mesoderm; *en*, endoderm; *v*, vitelline membrane.

2, 3. Anterior and posterior levels of a single 44-hour embryo; note greater concentration of phosphatase in ectoderm and mesoderm at anterior level. *h*, heart; *s*, somite; *lm*, lateral mesoderm.

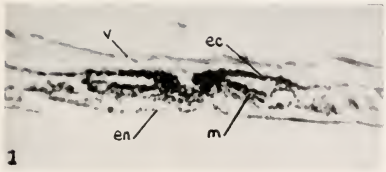
4. Through diencephalon of a 72-hour embryo; note greater phosphatase concentration in more ventral part of diencephalon.

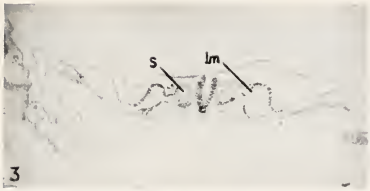
5. Through bulbus of a 5-day embryo; note high phosphatase content of valve and spongy lining tissue of bulbus. *m*, myocardium; *ct*, connective tissue; *a*, *a*, auricles.

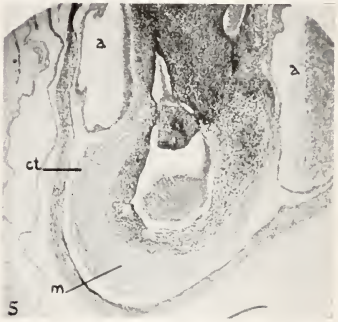
6. Through lung buds of a 5-day embryo. *a*, aorta; *b*, mesobronchus in lung bud; *o*, oesophagus; *l*, liver; *wd*, Wolffian duct.

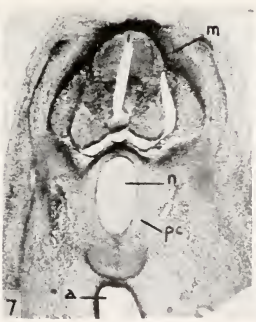
7. Differentiation of axial cartilage in fore-limb region of a 5-day embryo. *m*, phosphatase-containing membranous anlage of neural arch; *pc*, phosphatase-free protochondrium of future vertebral centrum; *n*, notochord; *a*, aorta. Note also phosphatase-free motor horns of spinal cord.

8. Liver diverticulum of 3-day embryo. *mg*, mid-gut; *l*, strongly positive liver diverticulum.









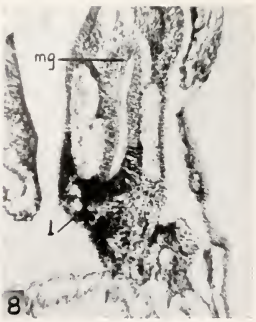


PLATE I

a minimal reaction, but more often they are negative so that there is usually a sharp line of demarcation between the embryonic and extra-embryonic tissues.

At 72 hours phosphatase is still distributed widely through the embryo, which continues to present a darkened appearance; but numerous tissues exhibit a lowered reactivity, evinced principally in the cytoplasm. This is especially true of the notochord at anterior levels, and the muscular wall of the heart.

The brain gives a strong phosphatase reaction in the most dorsal parts of the metencephalon and myelencephalon, but at about the level of the auditory capsule the hind brain is definitely less reactive (7 +) than the mid-brain, which is heavily blackened throughout. The infundibulum is moderately positive. The region of the diencephalon between the eyes appears much darker than the more dorsal region (Fig. 4), and this dorso-ventral gradation continues into the telencephalon. The dorsal part of the nerve cord and the neural crests react more strongly than the ventral part of the cord, but this difference disappears at more posterior levels, usually around the point where the amniotic folds are still open. The cranial ganglia show considerable deposits (10 +).

The eyes, the nasal placodes, and the outer faces of the auditory capsules are all strongly reactive (9 + to 10 +). The inner face of the capsule, and the lens, react weakly.

The muscular walls of the ventricle and atrium are negative, but the endothelial linings are positive. The bulbus gives a moderately strong reaction, while the walls of the ducti Cuvieri and the ventral aorta are blackened (9 +). This reactivity tends to persist in the aortic arches and dorsal aorta, but it fades in the latter as its walls become thinner toward the posterior end of the body. The cardinal veins show no particular reaction. The omphalomesenteric veins, where they enter the body, are lined with heavily reactive mesoderm, but this reactivity disappears as the vessels spread out over the yolk.

The pharynx wall is moderately positive, as is Rathke's pouch. The closely packed mesoderm in the visceral arches reacts to a lesser extent than the mesoderm of any other region. The thyroid is strongly positive. The laryngo-tracheal groove reacts noticeably, but the remainder of the alimentary canal still exhibits the low activity of the endoderm from which it is derived. The liver diverticula however are more positive than the tissue from which they arise (Fig. 8).

The mesoderm and loose mesenchyme are positive throughout the body, the re-

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PLATE II

9–15—alkaline phosphatase

9. Inner ear of 8-day embryo; note alternate light and dark staining patches of labyrinth wall. *c*, anterior semi-circular canal; *an*, acoustic ganglion; *my*, myelencephalon.

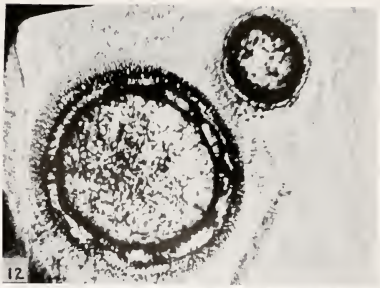
10. Optic chiasma of 8-day embryo. *oc*, central part of chiasma, just below optic nerves; *hy*, hypophysis.

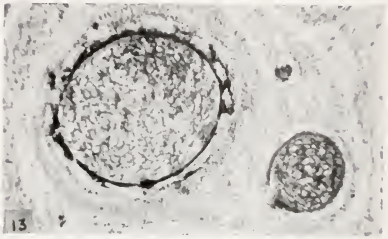
11. Longitudinal section through femur of 7-day embryo, showing thick coat of perichondral phosphatase in diaphysial region. (The dark band across the epiphysis is an artefact.)

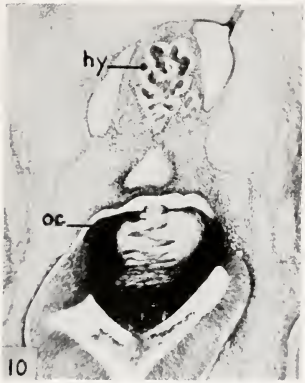
12. Transverse section through tibia and fibula of 8-day embryo. Note spongy nature of perichondral phosphatase layer, and also positive cells within the cartilage.

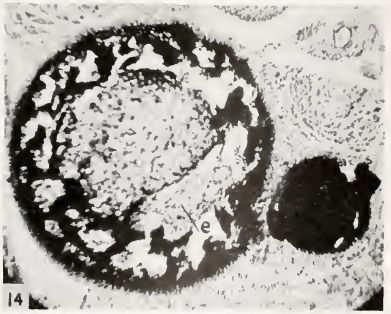
13. Control for 12 (opposite limb of same embryo). The stained material in the perichondral area is bone (calcium phosphate) deposited *in vivo*.

14, 15. As 12 and 13, for a 10-day embryo; section is near distal end of fibula. *e*, area of erosion.









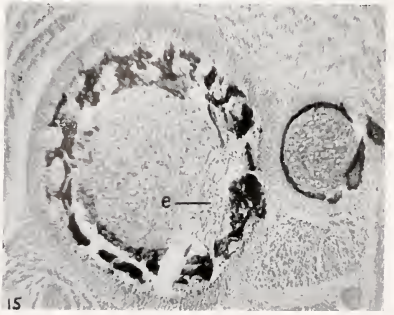


PLATE II

action still diminishing, however, toward the posterior end, as it does also in the neural tube. The nephric structures are darkened to the same extent as the surrounding mesenchyme.

Four to eight days

a. Nervous system

1. *The brain:*

Telencephalon.—The dorsal-ventral difference mentioned before is still evident in the cerebral hemispheres through the fifth day. On the ventral side there is a strongly positive region where the constriction between the hemispheres begins, but this disappears sharply where the partition is complete. Proceeding anteriorly one finds the outer and ventral faces on the hemispheres moderately positive (7 +), while the inner-dorsal faces are weakly so (4 +). The reaction gradually fades, so that the walls are almost negative near the olfactory part. On the sixth day the reactivity of the superior walls strengthens, and the phosphatase activity extends through the olfactory region.

On the seventh and eighth days the corpora striata and the pallium are lightly positive. A more intense reaction (7 +) appearing in the telencephalon medium however persists, somewhat attenuated, in the floor region of the hemispheres. The inner faces are virtually negative, as is the choroid plexus. The parencephalon is negative.

The epiphysis is negative when first formed, but phosphatase accumulates in the tubule cells as the glandular structure differentiates.

Diencephalon.—On the fourth day and later the ventral section of the posterior part of the diencephalon shows a strong reaction; but this diminishes just behind the eyes, so that for some distance between the eyes, as one proceeds anteriorly, only the central portion is strongly positive. The heavy stain at the entrance of the optic nerves, established earlier, persists; the nerves themselves are at first extremely reactive but their enzyme distribution becomes less uniform, for the differentiated supporting glia and connective tissue are negative, and only the nerve fibers retain the enzyme. As the thick walls of the thalamus develop, they show a reaction graded from moderate (5 +) dorsally to strong (9 +) on the floor. This

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PLATE III

19, 21—acid phosphatase
16–18, 20, 22, 23—alkaline phosphatase

16. Through neck of 8-day embryo. *t*, thyroid; *o*, oesophagus; *gn*, ganglion nodosum; *cc*, common carotid artery; *j*, jugular vein.

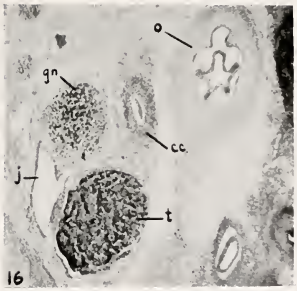
17. Part of myelencephalon of 7-day embryo. Note phosphatase-containing tissue around lumen, and sharp decrease in phosphatase content of axones from ganglion (*g*) as they enter myelencephalon.

18, 19. Proventriculus of 8-day embryo. Note phosphatase content of mesenchyme surrounding glands. *g*, gland; *cm*, circular muscle; *ca*, coeliac artery; *l*, liver. Note that with alkaline phosphatase liver cells are unstained and endothelium stained; with acid phosphatase cells are stained and endothelium unstained.

20, 21. Mesonephroi and associated structures of 8-day embryos. *gl*, glomerulus; *st*, secretory tubule; *ct*, collecting tubule; *md*, Müllerian duct; *wd*, Wolffian duct; *go*, gonad; *mt*, metanephric tubules and blastema; *r*, rib.

22. Blood vessel and positive endothelium in liver of 8-day embryo.

23. Positive endothelium in ventricle of 8-day embryo. *rc*, red cells.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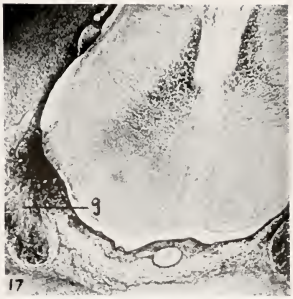
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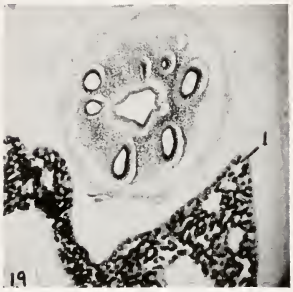
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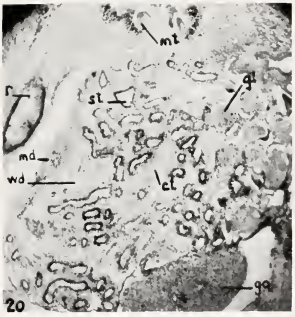
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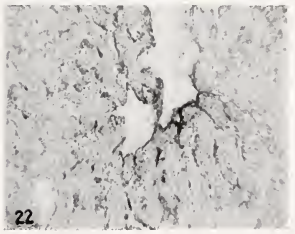
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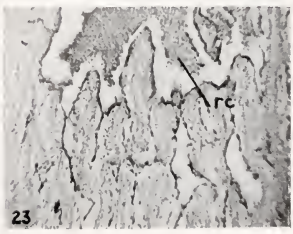
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PLATE III

intense reaction is continued around the lamina terminalis, and on the eighth day the optic chiasma (Fig. 10) shows extremely heavy deposits (12 +).

Mesencephalon.—A heavy deposit (8 +) in the outer wall of the more dorsal part of the optic lobes gives the slightly reactive inner tissue the appearance of being surrounded by a shell; with the persistence of this difference the outer region comes to include the layer of short radially arranged fibers that develop near the surface on the sixth day. The ventral and posterior walls of the aqueduct are markedly positive (7 +), the dorsal and anterior walls less so (4 +). This dorso-ventral gradient, as described above, continues forward into the diencephalon.

Metencephalon and myelencephalon.—At its junction with the mesencephalon, the metencephalon shares the high phosphatase concentration of the mesencephalic floor. On the fourth day this persists for a short distance posteriorly and then fades into a more moderate reaction which grows lighter on successive days. On the sixth day, however, heavy deposits (10 +) concentrate around the outer ventral corners of the fourth ventricle and taper off into the ventro-lateral tissue. This distribution continues backward, heavy deposits remaining close to the narrowing lumen while lighter deposits (7 +) gradually spread through the whole ventral region, except for the negative raphe (Fig. 17); as the spinal cord is entered, this pattern resolves itself into the cross-band of phosphatase-rich cells which has already been described (Moog, 1943a). The thin roof of the hind brain, and also the cerebellum, are negative.

2. *The cranial ganglia:*

Throughout the period under consideration all the cranial ganglia but the eighth show marked phosphatase activity (Fig. 16) of fairly uniform intensity (10 +). The axones connecting the ganglia to the brain are positive outside the brain and negative, or weakly positive, inside, the line of demarcation being extremely sharp (Fig. 17); the processes distal to the ganglia, however, are generally much less positive. Limited regions of the auditory ganglion stain darkly, but the bulk of the ganglion, including all those portions which amalgamate with auditory structures, are negative.

3. *The spinal cord:*

The spinal cord has already been reported on (Moog, 1943a). In terms of the scale used in this paper, the highest concentrations of alkaline phosphatase in the cord may be rated as 9 + to 11 +.

4. *The sense organs:*

The ear.—On the fourth day the auditory capsule, as before, exhibits alternate patches of lightly and darkly staining tissue; as development proceeds it becomes apparent that the lighter patches are the rudiments of the maculae and cristae (Fig. 9). These areas, like the branches of the auditory nerve that fuse with them, grow less reactive and on the eighth day are virtually negative. The rest of the labyrinth wall is strongly positive (10 +), as is the entire internal limiting membrane.

The saccus endolymphaticus is variably positive, though its glands stain very deeply (11 +). The walls of the semi-circular canals stain only lightly, but the inner lining is strongly positive.

The rudiments of the Eustachian tube and of the tympanic membrane and cavity at first share the phosphatase content of the tissues from which they form, but by the eighth day they become negative.

The eye.—The eye tissue on the fourth day has generally the darkly stained appearance characteristic of most of the brain at this time, except that the median face of the cup is somewhat lighter than the rest. The nerve tract, which inserts into this less positive region of the retina, is intensely positive (10+). The fibers of the lens core are negative, but their nuclei take a dark stain, as does the epithelial rim. Up to the eighth day the only change in the lens is that the cytoplasm of the epithelial rim loses its reactivity; the nuclei of both core and rim remain at least slightly positive throughout.

In the retina the diminution of phosphatase spreads toward the outer face of the cup on the fifth and sixth days, so that by the eighth day only the thin lenticular zone, the future iris, is markedly active. The bulk of the retina at this time is lightly positive, but the inner layer of optic fibers shows the same high phosphatase content as in the optic tract itself; and in addition there is a darkly staining layer between the ganglion cells and the base of the layer of Müllerian fibers. This reactive layer appears to include the inner plexiform fibers and also some very large cell bodies which may be the amacrine cells. In the formation of this layer both the histological differentiation and the corresponding phosphatase development spread simultaneously from the fovea outward. The mesenchymatous keel of the pecten is moderately reactive, and the budding eyelids are noticeably darkened.

The nose.—The whole nasal epithelium shares the high phosphatase content of its immediate surroundings on the fourth day, but this gradually diminishes, although the internal limiting membrane continues to stain deeply; the surrounding mesenchyme meanwhile also becomes less reactive (5+). The condensed mesenchyme from which the nasal processes and turbinals are derived exhibits black deposits (10+) on the seventh day, just before the cartilage begins to appear, and the nasal septum also shows phosphatase activity before the cartilaginous bar forms. As the cartilage differentiates the phosphatase again disappears.

b. Digestive system

On the fourth day the condensed mesenchyme of the visceral pouch region has a uniformly positive reaction. The lining of the mouth and pharynx stain darkly at this time, but later this epithelium loses some of its phosphatase content so that by the seventh day only the anterior and dorsal regions of the mouth lining are positive. The muscular mass of the tongue reacts moderately at the base at first, but the more anterior portion as well as the surface is only slightly positive. By the eighth day only the epithelium of the front component shows any reaction. The epithelial cords of the thyroid stain heavily throughout (Fig. 16), and the reaction is intrinsic rather than due, as one might suspect from Gomori's (1941a) results on adult mammalian thyroids, to the presence of preformed phosphate in the colloid.

The alimentary tract itself is at first moderately positive throughout, but its positivity declines steadily, with the diminution of phosphatase affecting the more posterior parts of the tract first. The reactivity of the oesophagus wanes after the period of occlusion, although it has a thin lining membrane that continues to stain heavily (Fig. 16). Between the fifth and eighth days a decreasingly positive re-

action extends through the duodenum, the lower parts of the intestine meanwhile becoming completely negative in the cytoplasm. The glands of the proventriculus include some of the positive lining membrane when first seen on the sixth day, but on the following day are quite negative (Fig. 18). On the eighth day the cytoplasm of the entire alimentary epithelium is negative, and even the nuclei, which

TABLE I

Relative phosphatase activities of digestive tract and derivatives

(For explanation of values, see page 53)

The endoderm generally has an alkaline phosphatase rating of 5+ at two to three days, acid phosphatase 1+. Mesoderm: alkaline phosphatase 7+, acid phosphatase 2+.

Tissue	Alkaline			Acid		
	4 days	6 days	8 days	4 days	6 days	8 days
Oesophagus—epith.	7+	5+	3+	1+	2+	2+
mucosa	8+	5+	4+	1+	1+	1+
Crop —epith.		4+	3+		2+	2+
mucosa		5+	3+		1+	2+
Stomach —epith.	5+			2+		
mucosa	8+			3+		
Proventriculus—epi.		4+	3+		3+	3+ (body) 5+ (glands)
muc.		7+	7+		1+	4+
Gizzard—epith.		5+	4+		2+	4+
mucosa		7+	4+		2+	2+
Small intestine—epi.	3+	2+	2+	2+	2+	4+
muc.	8+	7+	4+	2+	1+	1+
Large intestine—epi.	4+	2+	1+	2+	2+	2+
muc.	8+	7+	4+	1+	1+	trace
Rectum	3+	2+	2+	1+	1+	trace
Cloaca	1+	1+	1+	trace	trace	trace
Pancreas	4+	4+	3+*	2+	3+	3+
Liver cells	7+	5+	2+	4+	5+	6+
Liver endothelium	9+	10+	12+	trace	trace	trace
Ductus choledochus		3 to 8+	3 to 6+	2+	3+	4+
Thyroid	10+	12+	12+	3+	5+	7+
Trachea	8+	5+	4+	2+	3+	4+
Bronchi	8+	5+	4+	2+	3+	4+
Mesobronchi	8 to 3+	7 to 3+	2+	2+	3+	4+
Entobronchi		3+	3+		2+	2+
Mesoderm of lung bud	8+	7+	4+	2+	1+	1+

* In tubules, about 8+.

were originally strongly positive, retain only a small part of their former reactivity. The phosphatase deposits also disappear from the linings of the crop and proventriculus, but those of the gizzard and duodenum remain positive. The cloaca is negative throughout.

The closely packed mesenchyme surrounding the developing alimentary tract gives a moderately strong reaction at first, and this continues to be true on the fifth

and sixth days, as the mesenchyme becomes differentiated into a layer of loosely packed tissue (mucosa) immediately around the digestive tube, and a denser layer of circularly arranged cells (muscularis mucosa) outside the first. By the eighth day, however, the muscular layer has lost most of its phosphatase, except for small amounts in the nuclei, but the inner layer continues to be reactive (Fig. 18).

The pancreatic tissue, as it differentiates, becomes less reactive than the mesoderm in which it lies. On the sixth or seventh day, however, phosphatase begins to appear in the organ itself, and on the eighth day, when a glandular structure begins to develop, the tubules are strongly positive. The liver has a strong reaction at four days, but with the progress of differentiation the center of the mass of tissue first becomes negative, and this negativity gradually extends to the periphery; by the eighth day even the nuclei of the hepatic cells have only a trace of activity. During the same period, however, phosphatase develops in the endothelium lining the sinusoids, the reaction appearing first around the principal venous channels and then spreading through all the liver spaces (Fig. 22). The ductus choledochus is reactive within the liver, but becomes negative as soon as it emerges from the liver substance.

The trachea is strongly positive on the fourth day, but the reactivity wanes steadily. The more proximal portions of the bronchi react similarly, but it can be seen on the fifth day that the phosphatase content diminishes sharply at the bend between the anterior and middle mesobronchi, and again between the middle and posterior mesobronchi. Both trachea and mesobronchi have an extremely black inner lining at first, but this too gradually loses its enzyme content. The entobronchi are almost negative throughout.

If the trachea of the hatched chicken has the same high concentration of phosphatase in its ciliary border as Bourne (1943) has described for the trachea of the rat and guinea pig, the accumulation of phosphatase must occur after the eighth day.

c. Urogenital system

Although it differentiates from active mesenchyme, the cytoplasm of the Wolffian body is generally weakly positive on the fourth day. The only parts which show an intense reaction are the necks of the tubules, and the nuclei throughout. The cytoplasm of the duct itself is clear along its entire length. As the structure differentiates further, a high concentration of phosphatase accumulates in the brush borders of the secretory tubules, while the quantity of the enzyme diminishes simultaneously in the outer borders of these tubule cells. The collecting tubules, Bowman's capsules, and the glomeruli, as well as the Wolffian duct, have little or no phosphatase during this period. The Müllerian duct however stains quite darkly (Fig. 20). The metanephric blastema is moderately positive, but the tubules that differentiate from it are only weakly so; the blastema of the ureter, on the other hand, contains only a little phosphatase, and the tube itself shows no change in this respect.

The gonads are quite reactive when they first appear, but their phosphatase content diminishes steadily. The same is true of the suprarenals. Neither organ had assumed a pattern of phosphatase distribution, as the findings of Gomori (1941a) and Kabat and Furth (1941) suggest that they might at a later stage.

TABLE II

Relative phosphatase activities of the urogenital system

At 2 to 3 days: mesoderm from which Wolffian body is formed has alkaline phosphatase rating of 8+; acid phosphatase, 2+.

Tissue	Alkaline			Acid		
	4 days	6 days	8 days	4 days	6 days	8 days
Glomeruli	3+	3+	3+	1+	1+	1+
Bowman's capsules	5+	3+	3+	trace	trace	trace
Secretory tubules—walls	10+	6+	3+	4+	4+	4+
—brush border	10+	14+	14+	4+	4+	4+
Collecting tubules	4+	trace	trace	2+	2; 8+	2; 8+*
Wolffian duct	4+	3+	2+	8+	8+	8+
Müllerian duct		8+	5+		2+	2+
Metanephric blastema		8+	8+	2+	2+	2+
Metanephric tubules		4+	4+	8+	8+	8+
Ureter	4+	4+	3+	2+	2+	2+
Suprarenal (provisional cortex)	8+	6+	4+	1+	1+	2+
Gonad	10+	8+	6+	2+	3+	4+

* The phosphatase content of the tubule rises sharply at its entrance into the Wolffian duct.

d. Circulatory system

The heart.—The cardiac muscle continues to be completely free of alkaline phosphatase; even the nuclei, which stain lightly on the fourth day, become completely negative by the eighth day, except that the nucleoli are still reactive. Thus a clear-cut boundary appears in the bulbus between the negative heart tissue and the phosphatase-containing tissue external to it. The thin endothelial linings, which by the fifth day reach into the deepest recesses of the trabeculae, stain solid black (Fig. 23). High concentrations of phosphatase are also found in the septa, including the cushion septum, a development which is already foreshadowed on the third day by thickening of the intensely reactive epithelium around the valves. The thick spongy lining of the bulbus shares this positive reaction (Fig. 5); in fact, all the vessels entering or leaving the heart are lined with phosphatase containing tissue.

The blood vessels.—When first laid down, all the blood vessels share the high reactivity of the condensed mesenchyme that forms them (Fig. 7). As late as the eighth day the still poorly differentiated small arteries are reactive throughout, but the walls of the aorta and the larger arteries gradually become free of phosphatase as the muscular tissue differentiates. However the pulmonary arteries, and the aorta and its major branches (Fig. 18), are lined with highly reactive endothelium. The umbilical artery has virtually no phosphatase by the eighth day, while the omphalomesenteric arteries have a reactive endothelium where they branch from the aorta, but this becomes negative as they pass outside the body.

The veins are not generally reactive (Fig. 16). The ducts of Cuvier and the sinus venosus, however, and later the proximal portions of the venae cavae, possess

the same phosphatase-rich lining as the heart itself. Likewise the meatus venosus and hepatic veins are lined with the heavily staining tissue that runs through the sinusoids, and the omphalomesenteric veins remain moderately positive throughout the period studied. The larger umbilical vein is rather strongly positive. Except for the capillary bed of the liver, the walls of the capillaries are by the eighth day not more reactive than the tissues that form them.

The presence of alkaline phosphatase in vascular endothelia at the early stages at which it has been observed is of interest in relation to the question of the origin of endothelial phosphatase. It is sometimes held that endothelial cells pick up phosphatase from the serum. If Armstrong and Banting (1935) are correct, however, in their contention that bone is the source of serum phosphatase, it is clear that phosphatase in the endothelium of six- or seven-day embryos cannot be derived from serum; more likely the endothelia, like other tissues, produce their own phosphatase.

The spleen has a weak, uniform reaction through the eighth day, at which time it is still in an early state of differentiation.

e. The skeleton

The hind limbs from the sixth to the tenth day were studied in most detail; pairs of limbs were fixed on each day, plus pairs of feet for the eleventh day, one member of each pair being used for phosphatase demonstration and the other for the demonstration of phosphate deposited *in situ*. All specimens were sectioned transversely.

The limb buds on the fourth day are merely masses of condensed mesoderm which share the high phosphatase content common to mesoderm at this time (8 to 10+). On the fifth day each mass becomes differentiated into an inner portion, the rudiment of the cartilage, which is more condensed and highly reactive; and an outer portion, destined to form muscle and connective tissue, which is comparatively rarified and less reactive. As the precartilages become transformed into protochondrium, their phosphatase content diminishes sharply except at the periphery, where a thin layer of compressed cells remains positive (8+); within the mass the nuclei continue to show a fairly strong reaction, but the cytoplasm and developing matrix become quite negative. During the protochondrial stage the enveloping layer of phosphatase-containing cells increases in thickness without giving a more positive reaction; but with the appearance of true hypertrophic cartilage the perichondrium begins to develop the most intense reaction found in the early embryo (16+); the cells affected are the perichondrial osteoblasts.

As is true of histological differentiation, the accumulation of phosphatase begins, in the long bones, midway along the diaphysis, and spreads gradually toward the epiphyses; simultaneously the layer increases in thickness, becomes open and spongy (Figs. 12 and 14), and its inner edge invades the matrix raggedly. In the fibula phosphatase appears throughout the diameter of the shaft toward the distal end, but where typical epiphyses occur the phosphatase accumulation stops abruptly at the zone of flattened cells (Fig. 11). When the phosphatase-laden osteoid has achieved a thickness in the diaphysis roughly equal to half the radius of the enclosed cartilage, thin shells of bone are laid down near the inner surface of the layer (Figs. 13 and 15). From Table III, which summarizes the development of carti-

lage, phosphatase, and bone in the hind limb from the sixth to the eleventh day, it will be seen that bone deposition follows inevitably when a certain level of cartilage differentiation is reached; this is equally true for the tibia, in which the development of the cartilage and osteoid envelope is rapid, and for the proximal phalanges, in which hypertrophy and phosphatase accumulation are slow.

It was pointed out above that the first differentiation of cartilage from mesoderm is accompanied by a marked diminution of the primitive embryonic phosphatase in

TABLE III

Summary of cartilage, alkaline phosphatase, and bone formation

Day	Structure*	Condition of cartilage	Condition of perichondral phosphatase layer	Deposition of perichondral bone
6	tibia, fibula	precartilage	light (8+)	−
	femur	precartilage	light (8+)	−
	metatarsals	mesenchyme	—	−
7	phalanges	precartilage	light (8+)	−
	metatarsal 1	mesenchyme	—	−
	metatarsal 2–4	protochondrium	2-thin, compact (12+)	−
			3, 4-thick, compact (16+)	−
	tibia, fibula	hypertrophic	thick, compact (16+)	−
	femur	hypertrophic	thick, compact (16+)	−
8	phalanx 1	precartilage	light (8+)	−
	phalanx 2–4	protochondrium	thin, compact (12+)	−
	metatarsal 1	precartilage	light (8+)	−
	metatarsal 2–4	hypertrophic	thick, spongy (16+)	3, 4+; 2−
	tibia, fibula	hypertrophic	thick, spongy (16+)	+
	femur	hypertrophic	thick, spongy (16+)	+
9	phalanges	protochondrium	thin, compact (12+)	−
	metatarsal 1	protochondrium	thin, compact (12+)	−
	metatarsal 2–4	hypertrophic	thick, spongy (16+)	+
	tibia, fibula	hypertrophic	thick, spongy (16+)	+
	femur	hypertrophic	thick, spongy (16+)	+
10	phalanges	hypertrophic	thick, spongy (16+)	−
	metatarsal 1	protochondrium	thin, compact (12+)	−
11	phalanges	hypertrophic	thick, spongy (16+)	+

* The most advanced portion of the structure is considered.

the cytoplasm. Shortly afterward the nuclei in the zone where hypertrophy occurs also undergo loss of phosphatase, so that the cells of the cartilage beginning to hypertrophy are negative. Already on the seventh day in the tibia and femur, however, some of these cells develop a very intense reaction while other neighboring cells continue to be negative; at the same time the matrix begins to exhibit signs of phosphatase activity. The reactive cells seem to decrease in number through the tenth day, and they are most numerous midway along the diaphysis. Possibly these cells are engaged in synthesizing the phosphatase that becomes con-

centrated at the periphery, or they may be endochondral osteoblasts, for erosion has begun in advanced cartilages on the tenth day (Fig. 14).

The anlagen of the vertebral bodies and neural arches pass through the same stages of cartilage differentiation as do those of the long bones, at about the same time, although by the eighth day the hyaline cartilage of the axial skeleton has achieved an advanced state of differentiation only immediately around the notochord (Fig. 7). Each separate element is enveloped by a compressed layer of elongate cells which are more reactive than those within the cartilage; this layer resembles that which envelops the small-celled cartilage of the long bones, but it never has anything like the tremendous phosphatase activity of the perichondrium of ossifying long bones. The cell bodies are generally only slightly reactive, but in the anterior region on the eighth day a number of reactive cells appear among the negative ones in the center of each future vertebra, where endochondral ossification will begin several days later. In a few sections through the neck of an eleven-day embryo it was observed that both matrix and cells of the cartilage close to the notochord contained a very high concentration of phosphatase, while the remainder of the vertebral cartilage contained none of the enzyme; ossification had not begun.

No detailed study was made of the development of the girdles, but it was noted that on the eighth day the coraco-scapula is covered by a phosphatase-rich perichondrium (14 +) similar to that of the limb bones; the layer is thicker on the coracoid than on the scapular part. The clavicle at the same time is a mass of mesoderm heavily impregnated with phosphatase. The elements of the pelvic girdle show practically no perichondral phosphatase accumulation at eight days. The ribs however are heavily coated with strongly reactive phosphatase in the diaphyseal regions (Fig. 20).

The development of the skull was not studied.

f. Other tissues

Notochord.—The nuclei of the notochord are less intensely reactive on the fourth day than at earlier stages, and the cytoplasm is negative. Within the next two days the nuclei also lose the remainder of their phosphatase content.

Muscles.—As the fibers of the skeletal muscle differentiate, their cytoplasm loses entirely the phosphatase content of the primitive mesoderm from which they are formed. The nuclei lose most of their phosphatase, but even on the tenth day they are still slightly reactive.

Skin.—The superficial ectoderm is intensely reactive at the beginning of embryogenesis, but already by the fourth day the phosphatase content of the cytoplasm has diminished sharply, and by the seventh day even the nuclei are negative. At the same time the thick mesodermal corium has also become negative. This change is not uniform, but begins first in the dorsal and anterior regions of the body.

Mesenchyme.—The loose mesenchyme distributed throughout the embryo is positive (8 +) as late as the fifth day, but thereafter it gradually loses its enzyme content. On the eighth day this tissue is almost negative in the body, but in the head masses of it which are presumably concerned in the ossification of membrane bone remain strongly positive.

Feather germs.—Undifferentiated feather germs were found on ten-day hind limbs. They contained a core of positive mesoderm (8 +).

II. Acid Phosphatase

Acid phosphatase is widespread in the early embryo, but in virtually all cases in apparently far lower concentration than alkaline phosphatase. With Gomori's (1941b) unmodified technique only the Wolffian duct and some of the kidney tubules ever produced a black deposit; in all other organs the deposits, even after 16 hours' incubation, took merely a golden-brown stain of varying intensity. As has been noted before, the extent and intensity of staining varied considerably among different specimens of the same age, and in spite of the preparation of a large number of specimens it was not easy to decide how much of the variability is intrinsic and how much due to the troublesome lability of the enzyme. The use of ascorbic acid as an activator improved the uniformity of the results but, as will be explained later, it did not entirely correct the inconstancies. Several embryos even failed to produce any deposits at all, for no readily apparent reason. When deposits did appear, however, their distribution and relative concentrations in the separate organs and throughout the body as a whole were quite constant.

Acid phosphatase is commonly found in nuclei, but unlike the alkaline enzyme it occurs only in uniform distribution in the karyoplasm, which takes a diffuse stain; generally in reactive cells, however, there is a darkly stained cap, which may be the Golgi apparatus, closely applied to the surface of the nucleus (cf. Moog, 1943). Bourne (1943) found such caps stained for alkaline phosphatase in the nuclei of certain intestinal cells; they were not observed in the alkaline-stained cells of the embryo, but the heavy reactions of the nuclear sap and membranes may have obscured them. On the basis of the relative distribution of the enzyme in nucleus and cytoplasm the same three classes of tissues may be defined as were described for alkaline phosphatase; but in addition, in the differentiation of some tissues acid phosphatase disappears almost completely from the nuclei as well as from the cytoplasm.

The difference in the nuclear staining is one argument against the possibility that the acid enzyme of this study is merely residual activity at acid pH of the much stronger alkaline enzyme. And this possibility is completely eliminated by several other facts. First, the alkaline enzyme is entirely suppressed at pH 8. Second, at later stages the two enzymes assume distributions that differ in many important respects. Third, at early stages as well as later, the acid enzyme is inhibited by fluoride, which has been shown to have no effect on the alkaline enzyme. And last, as will be demonstrated in a subsequent section, the two enzymes are affected in opposite fashions by a variety of chemical substances.

The first day

In the unincubated blastoderm the smaller yolk granules, whether in the superficial layers of the yolk or enclosed within the cells, show a moderately high phosphatase content, while the deeper layers of yolk are less reactive; but as in the case of the alkaline phosphatase, proximity to the blastoderm rather than the nature of the yolk, seems to determine the enzyme content. The central area of the ectoderm is the most reactive part at this time (about 3 +); the reactivity of the cytoplasm wanes somewhat near the germ wall. The endoderm has little cytoplasmic phosphatase, although the yolk granules enclosed in the cells cause it to stain no-

ticeably. The nuclei are difficult to detect, and do not seem to be stained more deeply than the cytoplasm.

As the primitive streak differentiates, the ectoderm composing it becomes more reactive (4 +), and the demarcation between the central ectoderm and the less reactive periphery becomes sharper. The endoderm stains more intensely than in the gastrula stage, and the mesoderm is intermediate in enzyme content between the ectoderm and endoderm; as the yolk granules disappear it can be seen that in the mesoderm and endoderm the nuclei are more reactive than the cytoplasm, but in the ectoderm the intensity of the cytoplasmic stain obscures the condition of the nuclei (Fig. 1). The head process ectoderm and the notochord share the high phosphatase content of the primitive streak ectoderm as soon as they are laid down, while the surrounding extra-embryonic tissue continues to be only weakly reactive (1 +). The activity of the lateral mesoderm increases when the somites begin to form.

Two and three days

The nervous system continues to be the most reactive on the second day; the notochord is also quite reactive, but the other tissues of the body are markedly weaker (2 +), although their nuclei stain rather deeply. The extra-embryonic tissues become negative at a short but variable distance from their confluence with the body. The same antero-posterior gradient obtains as was noted in the case of alkaline phosphatase.

On the third day the spinal cord is as rich in phosphatase as before, and in the ependyma sometimes even more so (4 +). In certain regions of the brain, however, there is a sharp decrease. The hindbrain continues to be reactive, especially where it passes into the spinal cord, but the midbrain is largely unreactive, except in its ventral region, and the diencephalon too remains positive only on its roof and around the point where the optic stalks enter. Of the telencephalic vesicles only the anterior faces react. The nasal pits, optic cups and lenses, and otocysts continue to be positive, though less so than the spinal cord.

The loose mesenchyme is negative, but condensed aggregations of mesodermal cells remain lightly positive (2 +). There is a sharp increase concurrent with the appearance of the Wolffian body, however, and already at the end of the third day the Wolffian duct is quite rich in acid phosphatase (8 +). The endoderm continues to be weak except at the points of the liver and allantoic diverticula: the liver tissue is extremely reactive (4 to 5 +) when it first appears, and the allantois is equally reactive; the mesoderm of the allantoic bud shares the strong reaction of the endoderm.

Four to eight days

a. Nervous system

1. *Brain and cranial ganglia:*

The brain continues to show some acid phosphatase activity through the fifth day. At that time the mid- and hindbrain are about equally reactive (2 +), with the ependymal layer being more positive than the rest; otherwise there are no clear-cut local differences. The bulk of the diencephalon contains only a trace of acid phosphatase, but the dorso-lateral walls, and the ventral portion where the optic

nerves enter, are slightly more reactive (1 +). The cerebral hemispheres can also be rated at 1 + to 2 +, except that the partition which separates them is negative. On the sixth day the quantity of acid phosphatase in the brain is still slighter, and on the seventh and eighth days only with the aid of ascorbic acid could traces of the enzyme be detected. Similarly the acid phosphatase content of the cranial ganglia falls to a mere trace by the eighth day.

2. *Spinal cord:*

The general phosphatase activity described for the spinal cord (Moog, 1943a) is of the order of 2 + to 3 +, with the concentrations in the motor horns and ependyma being considerably stronger (4 + to 5 +). The activity of the large cells of the ganglia can also be rated at 5 +.

3. *Sense organs:*

The sense organs in their earliest expressions share the phosphatase content of the ectoderm from which they are formed. Their reactivity wanes rapidly, however, and by the sixth day they are all devoid of phosphatase, except that traces remain in the retina.

b. Digestive system

The condensed mesenchyme of the visceral pouch region exhibits only slight activity on the fourth day, and even this small activity wanes subsequently. The epithelial lining of the mouth is almost negative, but the inner surface of the pharynx and the laryngotracheal groove retain some acid phosphatase activity throughout this period. The thyroid stains heavily, according to the same pattern as with alkaline phosphatase.

The oesophagus gains somewhat in activity on the fifth or sixth day (Fig. 6), attaining a moderate phosphatase content which it shares with the crop; the inner lining membrane does not stain more deeply than the cells. The mesenchyme surrounding the oesophagus is uniformly weak, but that around the crop by the eighth day exhibits a relatively high phosphatase content in the innermost layer of compact tissue; the intermediate layer of loose mesenchyme and the outer layer of involuntary muscle fibers are much less active. The stomach and intestinal endoderm increase markedly in enzyme content up to the eighth day. The body of the proventriculus accumulates considerable phosphatase, and the glands become even richer (Fig. 19); the outer mesenchyme shows the same reactions as just described. The endoderm of the gizzard is also increasingly active, but its mesenchyme, which is less differentiated than that further anterior, is only slightly active on the eighth day. The small intestine shows the same relations as the gizzard, but the endoderm of the large intestine and rectum continue to be only weakly positive, and their accompanying mesenchyme contains only traces of phosphatase. The cloaca develops a very small enzyme content during this period.

The liver cells continue to gain in phosphatase content as the organ grows (Fig. 19), the increase proceeding from around the large veins to the periphery; the sinusoidal linings however do not contain the acid enzyme. The ductus choledochus is positive, but less so than the liver tissue surrounding it. The pancreas is at first

only slightly positive, but as in the case of alkaline phosphatase there is some accumulation of the acid enzyme as the glandular structure begins to differentiate.

The trachea and the respiratory tubes which spring from it undergo the same small increase in phosphatase content as occurs in the digestive tract generally between the fourth and the eighth day (Fig. 6), though the entobronchi remain less positive than the rest. The mesoderm of the lung bud, on the other hand, is only faintly reactive throughout.

c. Urogenital system

The Wolffian duct continues to be the most phosphatase-rich organ in the body (Fig. 6). The tubules are at first equally reactive throughout, but by the fourth or fifth day the secretory portions of the tubules begin to show a heightened reaction both in the brush borders and throughout the cells of the tubule walls; this is the same condition as Gomori (1941b) found in the kidneys of several adult mammals. The collecting tubules are definitely less reactive except at their entrance into the Wolffian duct, where they suddenly exhibit the intensity of the duct itself. The glomeruli are slightly positive, and Bowman's capsules have merely a trace of activity. The Müllerian duct is moderately positive (Fig. 21). The metanephric blastema is only lightly positive, but the tubules display very high activity, almost equal to that of the Wolffian duct, as soon as they appear. The ureter is weakly positive.

The gonads seem to possess a uniform overall phosphatase content during the period under consideration. But by the eighth day the enzyme is concentrated principally in the cortex and sex cords, the interstitial substance being much less reactive (Fig. 21). The suprarenal increases slightly in reactivity by the eighth day, but, as is also true of the alkaline enzyme, close examination fails to reveal differentially stained elements which might be the chromaffin cells.

d. Circulatory system

With the exception of the spongy lining tissue of the bulbus, which regularly demonstrates a small phosphatase content, all parts of the circulatory system have generally been found to be negative (Fig. 6). In one case, however, in which freshly prepared specimens were incubated for 18 hours in the presence of 0.01 M ascorbic acid, traces of acid phosphatase were found in all parts of the circulatory system; there appeared to be no noteworthy local differences. The red blood corpuscles are negative.

The spleen has a low phosphatase content throughout the eighth day.

e. Other tissues

The loose mesenchyme has only a trace of acid phosphatase activity, but the condensed masses of the tissue retain their phosphatase content until differentiation occurs. The membranous anlagen of the cartilages are thus somewhat positive (2 +) on the sixth day, but as the definitive cartilage appears it is negative, except for small amounts of phosphatase in the cell nuclei. The acid enzyme does not appear to have any further role in the differentiation of cartilage bones; its possible relation to the differentiation of membrane bones was not examined.

The striped muscles similarly lose practically all of their acid phosphatase content as they differentiate from reactive mesoderm.

The notochord is virtually negative by the fifth day, except for traces of phosphatase remaining in the nuclei.

The skin also loses most of its primitive reactivity by the sixth day, after which time all layers are negative, except, again, for slight reactivity in the nuclei.

Chemical Tests

The purpose of determining the effects of various chemical reagents on the activity of the embryonic phosphatases is threefold: first, to discover to what extent the embryonic phosphatases are identical with the phosphatases which have already been found in a variety of tissues and secretions; second, to find out whether each enzyme is a single entity or shows local variations; and third, to elucidate the differences between the two enzymes, and incidentally examine the possibility that any part of the weak activity of the acid enzyme is merely residual activity at acid pH of the stronger alkaline enzyme. Six-day embryos are most suitable for these purposes, since at this stage both alkaline and acid enzymes have to a certain degree assumed what appears to be their definitive distribution in partly differentiated organs, yet at the same time large amounts of both enzymes are still present in their primitive state in undifferentiated tissues. In order to secure a large number of comparable slides for treatment, a ribbon 8 micra thick was cut from the embryo and consecutive sections in groups of two or three placed on slides; in this way a series of 12 or more slides all showing practically the same tissues could be obtained. Such series were made at the eye, fore-limb, mid-body, and hind-limb level. Incubation was continued for 1½ hours in tests for the alkaline enzyme, 15 hours for the acid enzyme.

pH.—The alkaline phosphatase activity was weakened at pH 8.6 and abolished at pH 8.0. No acid phosphatase activity was obtained at pH 4.7 or pH 5.4.

Mg^{++}.—0.01 M $MgCl_2$ markedly activates the alkaline enzyme, at least doubling its production of phosphate during a one-hour run. A 0.001 M solution has a slighter effect. A 0.01 M solution does not affect the activity of acid phosphatase. These results are in agreement with the generally accepted hypothesis that Mg^{++} is a coenzyme of the alkaline phosphatase, but has no relation to the acid enzyme.

Mn^{++}.—The peculiar effect of $MnSO_4$ on the precipitate formed under the influence of acid phosphatase has already been reported (Moog, 1943b); it was concluded that no true activation is involved. The effect of $MnSO_4$ on the alkaline enzyme could not be tested, since its addition to the alkaline incubation solution causes the formation of a precipitate.

Zn^{++}.—Hove, Elvehjem and Hart (1940) reported that Zn^{++} increases the activity of crude alkaline intestinal phosphatase, decreases that of kidney and bone, and inhibits all three after dialysis. In the embryo 0.001 M $ZnSO_4$ greatly inhibits the activity of alkaline phosphatase, while 0.01 M abolishes the activity. Neither concentration had any effect on acid phosphatase.

F^-.—NaF is commonly accepted as an inhibitor of acid but not of alkaline phosphatase. 0.01 M NaF completely inhibited the acid enzyme in these studies; it could not be tested against the alkaline enzyme because of the very low solubility of CaF_2.

Sodium glycocholate.—Bodansky (1937) reported that various bile acids, including glycocholate, inhibit bone and kidney, but not intestinal, alkaline phosphatase; also Schmidt and Thannhauser (1943) found intestinal phosphatase to be unaffected by bile acids. In nine-day embryos 0.01 M Na glycocholate completely suppressed the activity of alkaline phosphatase in heart and liver endothelia, nerve and osteoid tissue, kidney and gonad, and also in the epithelium, brush border, and mucosal mesenchyme of the small intestine.

Phloridzin.—Lundsgaard (1933) originally showed that phloridzin inhibits both phosphorylation and dephosphorylation by kidney phosphatase. Later Kalckar (1936) and Beck (1942) substantiated the claim that phloridzin suppresses the phosphorylation of glucose by kidney extracts at neutral pH; but Beck also reported that phloridzin inhibits the hydrolytic activity of kidney phosphatase at pH 5 but has no effect at pH 7–9; the latter finding is in agreement with the results of numerous other workers, including Kritzler and Gutman (1941), who found that 0.01 M phloridzin has no effect on the alkaline phosphatase activity of the rat kidney in either the direct chemical or histochemical technique. The embryonic alkaline phosphatase, however, was very strongly (though not quite completely) inhibited by 0.01 M phloridzin, whereas the acid phosphatase was but slightly affected by the same concentration.

Reducing substances.—Albers (1935) presented extensive evidence to show that kidney phosphatase is inhibited by sulfhydryl compounds, and his results have several times been substantiated wholly or in part (del Regno, 1939; Pyle, Fisher and Clark, 1937; Schmidt and Thannhauser, 1943). In this study it was found that the alkaline phosphatase activity is suppressed completely by 0.01 M glutathione and by 0.01 M cysteine hydrochloride; and it is also inhibited to a marked extent (estimated as 50 per cent) by 0.01 M ascorbic acid. (Possibly rapid inactivation of ascorbic acid at pH 9.3 accounts for the incompleteness of this inhibition; however, Kiese and Hastings (1938) found that in runs lasting less than an hour 0.01 M ascorbic acid also had only a slight effect.) Acid phosphatase could not be tested with the two sulfhydryl reagents, since their addition to the acid incubating solution caused the appearance of a heavy precipitate. But 0.01 M ascorbic acid activated the acid phosphatase in freshly prepared specimens to an extent that seemed reasonable to estimate at 300 per cent.

From the effect of ascorbic acid it was first thought that the loss of acid phosphatase activity that histochemical preparations commonly undergo might be due entirely to oxidation. Therefore the effect of ascorbic acid on sections which had been standing at room temperature for one week was compared with that on freshly prepared sections. Activation occurred in the older material, but the level of activity was not as high as that of activated fresh preparations. Hence it appears that the spontaneous loss of acid phosphatase activity is not due principally to oxidation reversible by ascorbic acid.

Oxidizing agents.—The strong effect of ascorbic acid on acid phosphatase nevertheless indicated the advisability of determining the effect of oxidizing agents on the enzyme. Although Barron and Singer (1943) have reported that acid phosphatase is not inhibited by sulfhydryl oxidants, the activity of the enzyme in the presence of 0.01 M iodoacetic acid was tested: there was no clearcut inhibition. On the other hand, the activity of alkaline phosphatase was almost completely suppressed by 0.01 M iodoacetic acid.

It was then decided to try the effect of a less specific oxidizing agent. In the case of the alkaline enzyme, 0.01 M potassium ferricyanide exerted a limited but definite inhibition, estimated as 30 to 40 per cent. In the case of the acid enzyme, complete inhibition occurred. This inhibition was quite reversible, as was shown by exposing sections to incubating solution containing ferricyanide for two hours, and then transferring to plain incubating solution; the deposits produced were equal to those produced in control sections not exposed to the oxidizer. According to Sizer, both acid and alkaline phosphatase are inactivated by only such oxidizing agents as have E_h values between + 400 mv. and + 600 mv., the range in which 0.01 M ferricyanide probably falls; the fact that the E_h would be higher in acid than in alkaline solution may thus account for the more severe effect on the alkaline enzyme.

Taken together, these results with oxidizers and reducers offer little support for the view that sulfhydryl groups are important in the functioning of phosphatases. Particularly in the case of the alkaline phosphatase, that view does not seem reconcilable with inhibition by both iodoacetic acid and —SH compounds. The acid phosphatase moreover has now at least twice been shown to be insensitive to the effects of sulfhydryl oxidants.

The purposes of the chemical tests have been clearly fulfilled. In the first place, it has been shown that the two embryonic enzymes correspond well with the patterns that extensive research has delineated for alkaline and acid phosphatases generally. With one important exception, such differences as have been indicated can be explained as being due to differences in the state of purification of the enzymes; the exception is of course the action of phloridzin. Presence or absence of protective substances, coactivators, etc., probably cannot be called into account here, at least for the alkaline enzyme, for Kritzler and Gutman made histochemical tests of the latter on material prepared just as in this study, except that they used alcohol as fixative. The situation is the more difficult to understand because the kidney phosphatase has by the sixth day assumed what has been repeatedly shown to be its definitive distribution (Gomori, 1941a; Kabat and Furth, 1941; Krugelis, 1942), and furthermore may be assumed to be functional; yet the embryonic kidney phosphatases do not give the same response to phloridzin as other kidney phosphatases have been found to give.

Secondly, it has been amply demonstrated that the two embryonic phosphatases are separate substances. In addition to the fluoride sensitivity of the acid enzyme, it has been shown that the two enzymes differ, both in primitive and in differentiated tissues, in their responses to Mg, Zn, phloridzin, ascorbic acid, and iodoacetic acid.

Thirdly, no evidence has been found to indicate that each enzyme is other than a single entity. Taking into consideration the varying concentrations of the enzymes in different tissues, it may be stated that in no case was one tissue affected more or less than others by any reagent. This finding is in agreement with most current work, for no attempt to fractionate the phosphomonoesterases which Folley and Kay (1936) have classified as AI (alkaline) and AII (acid) has succeeded, except that of Bodansky, cited above, whose results may be due to factors extrinsic to the enzyme proper.

Lastly, no differences have been found between nuclei and cytoplasm in regard to the characteristics of either phosphatase. This result was partly to be expected from the fact that the nucleotidase which Levene and Dillon (1930) and others have found in numerous animal tissues is now accepted as being identical with the common alkaline phosphomonoesterase, with which Folley and Kay indeed classified it. The acid phosphatase of nuclei, on the other hand, has apparently been neglected; the evidence presented here certainly indicates that it does not differ from the cytoplasmic acid phosphatase.

Discussion

The most significant result to emerge from this study may be the fact that all primitive embryonic tissues, and especially all nuclei at early stages, contain phosphatase, both alkaline and acid. That phosphatase may occur in tissues which are far from assuming their definitive function has been demonstrated before by Kabat and Furth (1941), who found the substance in the mesenchymal anlagen of the leg and tail in the mouse embryo; the present results suggest that phosphatase may be an invariable concomitant of primitive tissue. It seems not unlikely that the function of this phosphatase may in part at least be concerned in cell division. In the chick embryo both alkaline and acid phosphatase are active in almost every tissue during the stage of intense cell proliferation; but there are exceptions. The extra-embryonic cell layers are very poor in both enzymes at the stage when they are expanding rapidly over the yolk, and the myocardium is all but devoid of phosphatase during a long period of rapid nuclear proliferation. Similarly, other workers have found that active tumors do not necessarily contain much phosphatase (Gomori, 1941b; Kabat and Furth, 1941). The traces of the enzymes that persist in the heart nuclei may of course be sufficient for the mitotic needs of the tissue. Willmer (1942) has indeed shown that in cells of chick heart tissue grown *in vitro,* alkaline phosphatase activity is intensified during mitotic activity and then regresses, leaving the cell negative except for reaction in the nucleolus and centrosphere. But if the very small amount of phosphatase demonstrated by Willmer, or observed in the sectioned heart muscle, is the actual requirement of mitotic activity, then a question remains: what is the function of the excess of phosphatase which the embryo possesses above this basic minimum? In the central nervous system, for example, phosphatase is not limited to the ependyma, where proliferation occurs; and even the undifferentiated endoderm, which is relatively weak in alkaline phosphatase, has a modest amount of the enzyme in its cytoplasm, and considerably more in the nuclei.

But proliferation is only one aspect of the function of primordial cells; equally important is the preparation for the histogenetic expression of differentiation. Experimental embryology has shown repeatedly that differentiation proceeds during a long period before its effects are fully realized, for there are a great number of cases in which transplants made at intervals from regions of apparent undifferentiation display increasing ability to manifest their normal prospective value; this is of course the phenomenon which Huxley and de Beer (1934) have termed "progressive chemo-differentiation." What then of the fact that all tissues of the early chick embryo contain substantial amounts of phosphatase during their period of chemo-differentiation leading to the assumption of definite form? It may not be without

significance that phosphatase is abundant in the primitive streak and its associated structures, and appears in the true embryonic structures as soon as they are laid down. Thereafter no tissue becomes nearly free of phosphatase until its definitive form is clearly indicated; this is equally true of cardiac muscle, which is almost free of the enzyme at the time it starts to beat; of the alimentary tract, parts of which become quite free of phosphatase as they become morphologically demarcated; of parts of the brain, of cartilage, etc. Moreover the increase of phosphatase which the hinder parts of the body undergo through the second day, when chemo-differentiation is proceeding actively, is suggestive in this regard, and so is the heightened phosphatase content of the liver diverticula between their appearance and the development of true liver tissue. The relatively high concentrations of acid phosphatase in the nervous ependyma, and the concurrent proximo-distal courses of histological differentiation and phosphatase decline in the developing retina, also seem pertinent. It is interesting, too, that Krugelis (1942), working with mouse testes, observed that the high concentration of alkaline phosphatase in the spermatogonia and spermatocytes declines to negativity as differentiation proceeds. For the time being, one can merely point out these suggestive parallels; but studies now contemplated will examine in more detail the possible interdependences between the progress of developmental patterns and the changes of phosphatase content.

The basic chemical role of phosphatase in development is even more obscure. Yet considering the tremendous importance which current research is attaching to phosphate transfer, it may not be extravagant to suggest that, among the diverse chemical mechanisms that lead to diverse histologies, there is during a considerable portion of primary differentiation an invariable factor involving phosphorylation and dephosphorylation, and so necessitating the presence of phosphatases. Perhaps the synthesis of proteins under the influence of phosphate-bearing nucleic acids involves phosphatase activity; it will be remembered that Caspersson and Thorell (1941) found especially high concentrations of nucleic acid in the cytoplasm of chick embryo cells in the earliest stages of development. This view of course would relate phosphatase activity to form change, rather than to histogenetic differentiation. In any case it will be interesting to learn why two phosphatases are simultaneously present in primitive tissues, and why their relative activities in different regions vary to some extent.

Although the chemical tests have shown no differences between phosphatases in differentiated tissues and in still undifferentiated anlagen, the phosphatases which accumulate in differentiated structures should probably be regarded as existing in a different phase from the primitive enzymes. The former, that is to say, are not merely remnants preserved from the early period, but are in themselves consequent on differentiation. A comparison of activities will illustrate this point. In the case of the alkaline phosphatase, the perichondrium, epithelia of heart and liver, brush borders of secretory tubules, and several other tissues show a level of activity higher than ever attained by the "unspecialized" enzyme of pre-differentiated tissues, and the same is true, in the case of acid phosphatase, of the Wolffian duct, liver cords, metanephric tubules, and so on. In all these tissues histological differentiation seems to entail somewhere in its course a chemical differentiation in the sense of production of phosphatase to be used in the incipient functioning of the organ. Thus the mesonephros, which is functioning already on the fifth day (Boyden,

1924), assumes by that time a phosphatase distribution virtually identical with that which has been described, especially in the case of alkaline phosphatase, for the adult mammalian kidney (Gomori, 1941a, b; Kabat and Furth, 1941). But the alimentary tract, non-functional in the embryonic period, loses much of its primitive epithelial alkaline phosphatase after becoming well-launched on its course of definitive differentiation, and it does not accumulate more up to the eighth day, even though in adult chickens, as well as mammals, parts of the digestive mucosa are extremely rich in the enzyme (cf. Folley and Kay, 1936; Gomori, 1941a; Kabat and Furth, 1941). And again, in organs which do not have one or both enzymes in their adult condition (e.g., the myocardium, or the liver cells in respect to alkaline phosphatase) there is usually a swift disappearance of the enzyme after the first stages of differentiation are completed; though of course it is not contradictory that a few rather well-differentiated organs (e.g., the brain, which is poor in phosphatase in its adult condition) contain phosphatase which has no obvious functional role; in such cases the enzyme may play a part in the more advanced stages of differentiation. Thus the specific organ phosphatases accumulating as differentiation proceeds are not necessarily conterminous in origin with the primitive phosphatases of the first days, and it will probably be profitable to consider them as separate entities.

This concept of a diphasic phosphatase occurrence in young embryos agrees well with the results of Lipmann (1936), who found a peak of alkaline phosphatase content in extracts of whole chick embryos on the sixth day. The histochemical preparations certainly give the impression that there is such a peak between the fifth and seventh days, for at that time the specific organ phosphatases are accumulating rapidly while the phosphatase in undifferentiated tissues, especially mesoderm, is still present in great quantities, although it disappears very soon thereafter with the appearance of cartilage and skeletal muscle.

Among the separate observations reported, those bearing on the relation of phosphatase to ossification deserve to be commented on. Robison's hypothesis that the activity of alkaline phosphatase is a causative factor in the deposition of bone salts is now generally accepted, and my results on the long bones are in full agreement with it. These results demonstrate, as Fell and Robison (1929) have already shown for chick femora cultivated *in vitro,* that the extremely high phosphatase concentration which is associated with ossification begins to accumulate with the differentiation of hypertrophic cartilage, and that the phosphatase concentration from seven to ten days is largely confined to the perichondrium, where it is limited to the diaphysial region between the zones of flattened cells—the exact region in which bone is seen to be deposited. Further, it is clear that in all bones of the hind limb the deposition of calcium phosphate is preceded by the development of a broad zone of high phosphatase activity. This phosphatase seems to be derived from the perichondral osteoblasts in which it appears. It is true that the body of the cartilage contains reactive cells, but the low activity of the matrix indicates that these reactive cells do not, within the period of observation, eject their phosphatase, as Bourne (1943) showed that similar cells do in cartilage formed in holes drilled in adult bone. Rather the resemblance between these positive cells of the long bones and the phosphatase-rich endochondral osteoblasts of the future

vertebral bodies suggests that the former are also of endochondral function, and are not concerned in the production of perichondral phosphatase.

The data as presented in Table III demonstrate that the state of differentiation of the cartilage, the quantity of phosphatase accumulated, and the amount of calcium phosphate deposited, are interrelated. In every bone examined, it was found that the concentration of phosphatase rises at the same rate as the cartilage hypertrophies, and that when the phosphatase-rich osteoid reaches a certain thickness, bone begins to be laid down; this is equally true in the tibia, where the entire process is extremely rapid, and in the proximal phalanges, which remain for two days in the protochondrial stage, and do not commence ossification until the eleventh day. In no case was any one condition found to be out of phase with the others. Fell and Robison (1934), investigating Robison and Rosenheim's (1934) postulated second mechanism of ossification, found that this mechanism, the ability of a tissue to deposit bone salts from a supersaturated solution, develops gradually during the period when bone is normally deposited. Evidently this development is paralleled by the accumulation of perichondral phosphatase.

Recently Horowitz (1942), using the histochemical technique, has shown that in fetal rat heads of thirteen days or more glycogen and phosphatase are simultaneously present only in tissues that ossify, and he has thus given fresh impetus to the view, originally suggested by Harris (1932), that glycogen plays a role in calcification, being possibly the source of the required phosphoric esters. Considering Glock's (1940) report that in rat bones taken a few days before birth glycogen is most concentrated at the primary center of ossification, it might be of interest to know whether in the very early material investigated in this study the accumulation of glycogen also parallels the cartilage-phosphatase-bone correlation which has been observed. It is worth noting that, according to Dalton (1937), glycogen first appears in the chick liver on the seventh day, increases slightly, and then decreases between the ninth and thirteenth days, the very period in which ossification is proceeding most actively. Moreover, alkaline phosphatase vanishes from the liver cells just before glycogen begins to be stored there.

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Summary

1. Both acid and alkaline phosphatases are present in the unincubated blastoderm of the hen's egg, and in all embryonic tissues during the first two or three days of development. The concentration of alkaline phosphatase is much greater than that of acid phosphatase.

2. Phosphatase persists as long as a tissue remains undifferentiated. As differentiation proceeds, phosphatase in some cases disappears and in others accumulates in higher concentration than in the primitive phase. Alkaline phosphatase is more widespread than acid.

3. The changes in phosphatase distribution in the principal soft organs up to the eighth day, and the relation of alkaline phosphatase to bone deposition in the hind limb up to the eleventh day, are considered.

4. The effects of a variety of chemical agents on both phosphatases are reported.

5. The possible significance of phosphatases in the processes of embryogenesis is discussed.

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A QUANTITATIVE SURVEY OF THE INVERTEBRATE BOTTOM FAUNA IN MENEMSHA BIGHT

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Extensive qualitative surveys of the marine invertebrate bottom dwelling organisms in the Woods Hole area have been carried out by Verrill (1873), Sumner, Osborn, and Cole (1911), Allee (1923), and several other investigators, but quantitative methods have not been used in the study of the fauna of this region. A study was therefore begun in order to examine in a quantitative manner the more common marine invertebrate organisms occurring within a restricted area of the bottom of the Woods Hole region.

For the purposes of this evaluation, it was decided to examine the bottom fauna of Menemsha Bight. There are two primary reasons for this choice. In the first place, Menemsha Bight is a relatively well defined body of water formed by an indentation of the western shore of Martha's Vineyard island between Gay Head and Cape Higgon, near the western end of Vineyard Sound (Chart 1). Secondly, the flounder which are captured in the Bight during the months of July, August, and September are of considerable economic importance. Therefore, it is of interest to know in what manner the bottom fauna enters into the general food cycle of the area. Either the direct or the indirect food relationships of this fish may be influenced by changes in the number and type of smaller animals living on and in the bottom. This may in turn affect the length of season when the fish are present in the region and perhaps underlie annual fluctuations which occur in their abundance.

The present survey is a preliminary attempt to investigate these problems in a quantitative manner. In addition to determining the various common species of animals present in the bottom fauna, an attempt is made to establish their numerical distribution over the area. The wet weight of each catch and dry weight of the combustible organic matter that it contained are also determined as a rough index of the possible potential food supply which the different categories of animals represent.

[1] Contribution No. 330.

[2] It is a pleasure to thank Dr. George L. Clarke for his aid in this problem. His stimulating interest was a source of much encouragement to the author.

Quantitative studies of the invertebrate marine bottom fauna in flounder fishing grounds have been made by several European investigators. The work of Petersen (1911, 1915, 1918), Jensen (1919), Blegvad (1914, 1925), and others in the Danish fishing grounds is well known. Davis (1923, 1925) has made several quantitative studies of the bottom fauna in the deep water fishing areas of the North Sea, while Idelson (1930) has carried out comparable investigations on the Spitzbergen

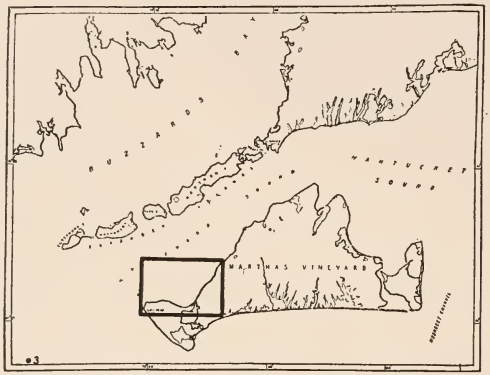


Chart 1. Map of Vineyard Sound and the adjacent islands. Menemsha Bight is the area enclosed by a heavy black line on the western end of Martha's Vineyard.

Banks. In the western hemisphere, however, the extensive quantitative studies of marine bottom fauna have been restricted chiefly to the Pacific coastal regions (Shelford and Towler, 1925; Shelford, Weese, *et al.*, 1935; Weese and MacNab, 1930) while the several investigations of bottom animals along the Atlantic coastline have been largely of a qualitative nature.

Methods

Apparatus

Many previous investigators have found that the Petersen dredge (Petersen, 1911) was the most satisfactory bottom sampler devised to date for the purposes of their work. Preliminary trials of this instrument on sand, mud, gravel, and stony bottoms in Vineyard Sound, however, reveal that this dredge is highly unsatisfactory for quantitative sampling in this region, for it is relatively small and due to its lack of weight the jaws of the bucket frequently fail to penetrate the bottom deeply enough to obtain a sample or to capture other than organisms living upon the surface of the bottom. For these reasons, a larger and heavier dredge of the so-called "clam shell" design was purchased from the Hayward Company of New York. This instrument, made of special rust-resistant steel, weighs over 300 pounds when empty and covers a section of the bottom 101×56 cms. in area with its jaws open. It digs to a maximum depth of 23 cms. and holds 56 liters of material (wet sand) when level full. The single cable used in operating the bucket is tightly fastened

to and winds about a counter-balanced revolving drum in the frame of the instrument. When the cable is hauled taut, the drum rotates and closes the jaws of the bucket. The drum turns in the opposite direction and opens the jaws when strain is removed from the closing cable. Thus a releasing hook inserted into the cable at a suitable point supports the weight of the instrument, with the terminal closing cable slack and the jaws open, during the descent to the bottom.

When removing animals from samples of bottom material of the size which this dredge obtains, manual sorting of the entire contents is impractical. Therefore, the material was poured into a hopper provided with a set of three removable screens which had pore spaces of 18.0, 10.0, and 1.8 mm. respectively. The sample was washed down through the screens, each of which withheld a fraction of the catch and thus prevented the coarser material from collecting in the final screen to crush the smaller, more delicate, organisms. A comparable method of removing animals from samples of bottom material has been used with success by Petersen (1911, 1913), Blegvad (1925, 1928), and Davis (1923, 1925).

The plan of the survey

It was considered impractical, due to strong tidal currents in Menemsha Bight, to attempt a grid-work of stations over the area. Instead, the stations were made in a series of five profiles extending offshore from the 2.5 meter depth contour along a compass course, with the samples taken at definite intervals. Two additional lines of stations were made roughly parallel to the shoreline (Chart 2). The close spacing of stations along such profiles gives more accurate information on the variability of bottom types and faunal aggregations than would be obtained from a series of stations scattered over the area; and it also permits ready location and resampling at any desired station. This "contour" method has been employed by Davis (1923) who found it especially useful where the organisms existed in patches or restricted regions of the bottom.

TABLE I

Variation among 13 20-liter samples taken at 4-meter intervals in Zone 2

	Average number	Standard deviation
Total number of species	14	24%
Clymenella torquata	12	26%
Ampelisca macrocephala	19	18%

In order to establish the variations in catches which might be due to the sampling process, 13 samples of comparable size (20 liters in volume) were taken from a uniform area (Zone 2) at intervals of only 4 meters. It was found that the total number of species in each catch varied 24 per cent from the average of 14 per sample. The number of *Clymenella torquata* captured were 12 per average sample, with a standard deviation of 26 per cent; and the number of *Ampelisca macrocephala* averaged 19 per sample, with a standard deviation of 18 per cent (Table I). It is possible that these quantitative differences in the catch were due to an actual variation in the distribution of the animals over the bottom. In view of the restricted size of the region tested, however, it is assumed that the variations were caused by the sampling process itself. The average quantitative differences be-

tween the faunas of the various zones are much greater than these standard deviations of the catches in a restricted uniform area (Tables II and III). Therefore, it is believed that the quantitative data are reliable. Throughout the survey, single samples were taken at each station. Deevey (1941) found that the use of single sample stations offered satisfactory results in his studies of Connecticut lakes.

Analysis of material

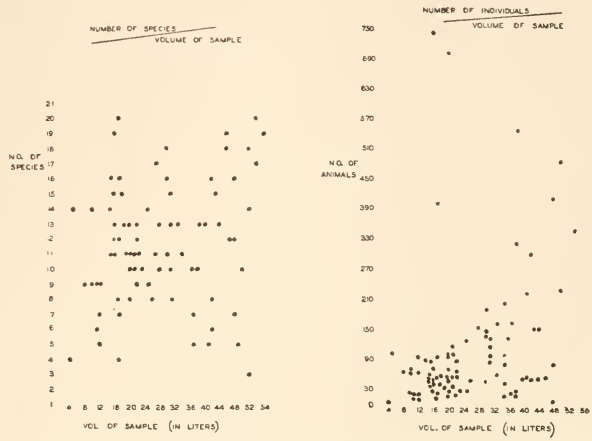
The catch from each station was brought into the laboratory where the organisms were identified and counted. The carapace of each large crustacean and the shells of all molluscs were removed and the wet weight of each catch was recorded (Davis, 1923; Berg, 1938). The estimated weight of all echinoderm tests was also subtracted from the weight of animals captured. The dry weight of the entire catch was obtained by drying the organisms to constant weight at approximately 120° C. The dried material was then ashed at approximately 650° C. By deducting the ash weight from the dry weight, an expression of the combustible organic matter present in the catch was obtained (Table III). The determination of organic content by this method is a customary procedure (Petersen, 1911; Juday, 1921; Birge and Juday, 1934).

Method of presentation of data

As in previous investigations of this nature by other investigators (Juday, 1921; Berg, 1938) it was found that the amount of bottom material obtained in the samples varied considerably, depending upon the relative hardness of the bottom and other conditions which affected the depth to which the dredge penetrated the bottom. However, there was no correlation between the size of the sample and the number of different species caught (Graph 1). In general, the larger samples of bottom material contained the greatest number of organisms (Graph 2). These facts support the assumption that the animals tend to be relatively uniformly distributed in the bottom material, at least down to the levels of the deepest grab of the dredge.

The invertebrate bottom organisms may be distributed vertically in the bottom sands in three different ways: 1. they may be restricted to a level at the surface of the bottom; 2. they may be uniformly distributed vertically throughout the bottom (down to the deepest grabs of the dredge); or 3. they may exist in greatest abundance at a level beneath the surface of the bottom material. If the first situation occurs, the quantitative data on the distribution of the organisms are best expressed on an area basis. This method has been used widely in quantitative marine studies, in spite of the variations in size of the samples obtained and although the vertical distribution of the organisms is not established. If the second situation obtains, the data should be expressed on a volumetric basis. This would eliminate variables introduced by samples of differing volume. If the third situation occurs, the choice between the area and the volumetric basis depends largely upon the numbers of animals existing between the levels of their greatest concentration and the surface of the bottom. In the present survey, the data presented in Graphs 1 and 2 support the second assumption. Therefore, the quantitative values for numbers of animals, wet weight of the catch, and the dry weight of the organic content obtained at each station have been calculated and expressed on the volu-

metric basis, using 20 liters of bottom material as the standard unit since most of the samples were of this size. The relative significance of the catch from samples of less than 10 liters or more than 40 liters in volume, when compared to that from samples more nearly approximating the standard size, is regarded as questionable, due to the large differences in volume. The data from such samples are not treated. The factor necessary to convert the data to expressions of the standard volume varied between the limits 0.5 and 2.0. Thus possible errors, should the uniform



GRAPH 1. The total number of species obtained in each sample, plotted against the volume of the sample in liters. It is apparent that there is no correlation between the size of the sample and the number of different species captured.

GRAPH 2. The total number of animals of all species in each sample, plotted against the size of the samples. In general, the larger samples contained the greater number of organisms.

vertical distribution of organisms not occur at certain stations, would be relatively small in comparison with the variations in abundance of animals from station to station and from zone to zone.

In making comparisons with other investigations, it should be remembered that this standard unit (20 liters) represents a portion of the ocean bottom 0.5 sq. meters in area, and 10.0 cms. in maximum depth. It is assumed that organisms in the bottom material below this level are of questionable importance as components of fish diet.

Inclusion of the weight of large bivalve and gastropod molluscs in the data, in view of their probable insignificance as fish food (Blegvad, 1925), would produce distortion of the results if one wishes to determine the amount of food material available to bottom-dwelling fishes. Therefore, in order to give complete values for the catches in each zone, as well as to indicate their relative significance as a source of food to fishes, the data for each zone have been expressed in two ways: 1. with all organisms included (shells and estimated weight of echinoderm tests re-

moved); and 2. with the weights of large molluscs (*Cyprina islandica, Polynices immaculata*), molluscs possessing thick shells (*Venericardia borealis, Astarte* sp.) and all echinoderms omitted. A somewhat comparable method of expressing data was used by Berg (1938) who presented his results with all organisms included, and also with molluscs omitted.

Results

The ocean floor in Menemsha Bight can be divided into five major zones arranged approximately coincident with definite depth contours. Each zone is characterized by a distinct type of bottom material, and a distinctive faunal aggregation is associated with four of these types of bottom. Table II contains a list of the

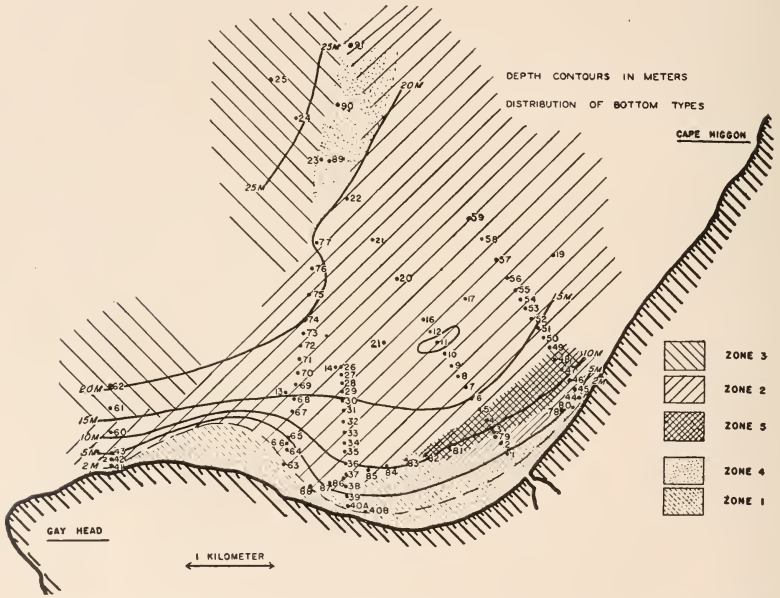


Chart 2. The number and location of each station, and the extent of the five zones in Menemsha Bight. The depth contours, in meters, are indicated by solid lines, with the exception of the 2-meter contour which is shown by a line of dashes.

catch at two stations in each zone, per standard volume of bottom material. Table III shows the predominant types of bottom (and average values for the catches) in each zone. Table IV is a list of the most common animals found in the bottom sands of Menemsha Bight and their average number in standard samples from each zone.

Table II

Typical catches from each zone

Zone 1.

Station 40-B

Clymenella torquata	1 specimen
Glycera dibranchiata	6 specimens
Nephthys incisa	1 specimen
Ninoe nigripes	1 specimen
Scolopolus fragilis	3 specimens
Tellina tenera	10 specimens

Station 41

Ampelisca macrocephala	1 specimen
Echinarachnius parma	1 specimen
Emerita talpoida	21 specimens
Nephthys bucera	1 specimen
Tellina tenera	19 specimens

Zone 2.

Station 26

Astarte undata	3 specimens
Clymenella torquata	27 specimens
Gammarus locusta	6 specimens
Glycera dibranchiata	6 specimens
Lumbrinereis hebes	1 specimen
Maldane sp.	3 specimens
Unciola irrorata	4 specimens
Venericardia borealis	1 specimen

Station 27

Ampelisca macrocephala	2 specimens
Clymenella torquata	9 specimens
Dolichoglossus Kowalevski	1 specimen
Gammarus locusta	3 specimens
Glycera dibranchiata	5 specimens
Lumbrinereis hebes	1 specimen
Maldane sp.	2 specimens
Nephthys incisa	1 specimen
Nephthys bucera	1 specimen
Ostrea virginica	1 specimen

Zone 3.

Station 24

Ampelisca macrocephala	31 specimens
Callocardia morrhuana	3 specimens
Cirolana concharum	2 specimens
Clymenella torquata	1 specimen
Cyprina islandica	2 specimens
Echinarachnius parma	12 specimens
Nassa trivitata	1 specimen
Polynices immaculata	1 specimen
Scolopolus fragilis	1 specimen
Unciola irrorata	1 specimen
Venericardia borealis	4 specimens

Station 25

Ampelisca macrocephala	22 specimens
Astarte quadrans	1 specimen
Cirolana concharum	1 specimen
Cyprina islandica	1 specimen
Echinarachnius parma	1 specimen
Marphysa sp.	1 specimen
Nephthys bucera	1 specimen
Polynices immaculata	1 specimen
Scalibregma sp.	1 specimen
Venericardia borealis	3 specimens

Zone 4.

The fauna of this zone was essentially similar to that of zone 3, in its quantitative and qualitative aspects.

Zone 5.

Station 48

Amphitrite ornata	1 specimen
Astarte castanea	2 specimens
Astarte undata	1 specimen
Asterias forbesi	2 specimens
Callocardia morrhuana	1 specimen
Cancer irroratus	1 specimen
Chaetopleura apiculata	2 specimens
Cirratulus grandis	1 specimen
Crepidula fornicata	692 specimens
Lepidonotus squamatus	1 specimen
Marphysa sp.	1 specimen
Pagurus longicarpus	1 specimen
Phascolosoma gouldi	2 specimens
Pinnixia cylindrica	1 specimen
Trophonia affinis	10 specimens
Venericardia borealis	19 specimens

Station 81

Arabella opalina	1 specimen
Arca transversa	2 specimens
Callocardia morrhuana	3 specimens
Cancer irroratus	1 specimen
Chaetopleura apiculata	1 specimen
Crepidula fornicata	353 specimens
Gammarus locusta	1 specimen
Lepidonotus squamatus	1 specimen
Libinia emarginata	1 specimen
Nereis virens	1 specimen
Pagurus longicarpus	7 specimens
Phascolosoma gouldi	1 specimen
Scalibregma sp.	1 specimen
Trophonia affinis	16 specimens

TABLE III

The wet weight of the catch, and the dry weight of the organic matter it contained, in the average standard-sized samples from each zone

Zone	Most numerous animals	Average wet weight of the catch in 20 liters of bottom material		Average dry weight of the organic content of the catch in 20 liters of bottom material	
		A[3]	B[4]	A[3]	B[4]
1	*Emerita Tellina Ampelisca*	5.6 gms.	1.9 gms.	0.9 gms.	0.33 gms.
2	*Clymenella Ampelisca*	27.2 gms.	10.0 gms.	2.6 gms.	1.3 gms.
3	*Ampelisca Cyprina*	164.0 gms.	9.8 gms.	29.4 gms.	1.0 gms.
4	*Ampelisca Echinarachnius*	73.0 gms.	6.3 gms.	10.7 gms.	0.5 gms.
5	*Crepidula*	364.0 gms.	360.0 gms.(?)	36.2 gms.	30.9 gms.(?)

Zone 1

Between the 2.5- and the 5-meter depth contours, the bottom is of a medium coarse yellow sand, containing a few small stones of pea size. An average standard unit of bottom material from this zone possessed five different species and a total of 32 animals of all species. *Emerita talpoida* and *Tellina tenera* were the most numerous organisms in this zone. The location of stations in this as well as in remaining zones is shown in Chart 2.

Zone 2

The coarser sands of Zone 1 graded rather abruptly (as shown by observations at stations 63–64; 38–39; 1–2; etc.) into fine mixed sands of white, yellow, and black color. This bottom material, called "Zone 2," was situated largely between the 5- and 20-meter depth contours, and extended over 80 per cent of the floor of the Bight. Annelids, small amphipods, and other organisms of known value as food for fishes were most numerous in this region, with *Ampelisca Macrocephala* and *Clymenella torquata* occurring in greatest numbers (Charts 2 and 4). The larger molluscs, however, such as *Cyprina islandica* and *Callocardia morrhuana* were more prominant by virtue of their relatively greater weight. The stations from this region which are shown in Table II illustrate the fact that the number of animals of a single common species (*Clymenella torquata*) may fluctuate 50 to 80 per cent from the average value in adjoining stations, when the number of such individuals caught is relatively low. It is seen in Table III that this zone was characterized by a heavier catch of organisms known to be of value as food for bottom dwelling fishes, than were the remaining zones.

[3] Including all organisms.

[4] With the weight of large molluscs, molluscs with thick shells, and the estimated weight of echinoderms omitted.

The number of species taken in samples from this and from the remaining zones varied from six or seven at stations 32, 33, and 34, to 19 or 20 species at stations 76, 77, 21, 70, 71, 11, 58, 59, and 52. In general, however, the regions containing the greatest number of different species were found in Zone 2. By comparing Chart 3 with Chart 4, it is evident that these regions are also generally character-

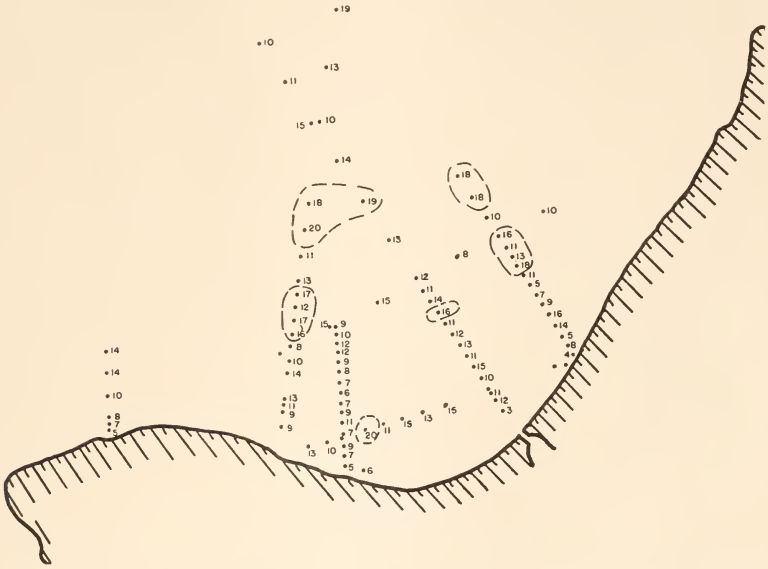


CHART 3. The number of different species captured at each station. A line of dashes encloses each region where the number of species obtained was greater than 15/sample.

ized by a great number of Ampelisca and Clymenella. In other words, in Menemsha Bight the individuals of a common species are generally more abundant in those areas where a large number of different species are found. Some of the possible factors which may determine the distribution of these organisms in Menemsha Bight will be treated in the discussion.

Zone 3

Beyond the 20-meter depth contour, the bottom was composed of loose, coarse sand, as shown by observations at stations 62, 24, and 25, which also contained shell fragments and small pebbles of various sizes. This zone contained a mixed fauna with amphipods and annelids present in each sample (Table II) but with no single species attaining numerical prominence. The large bivalve molluscs, such as Cyprina and Callocardia, composed the great bulk of each catch. It is interesting to note that *Cirolana concharum* and *Astarte quadrans* were found only at stations

in this zone; whereas several organisms (*Echinarachnicus parma, Unciola irrorata, Scolopolos fragilis*) were also found in the coarse sand bottom of Zone 1, at much shallower depths.

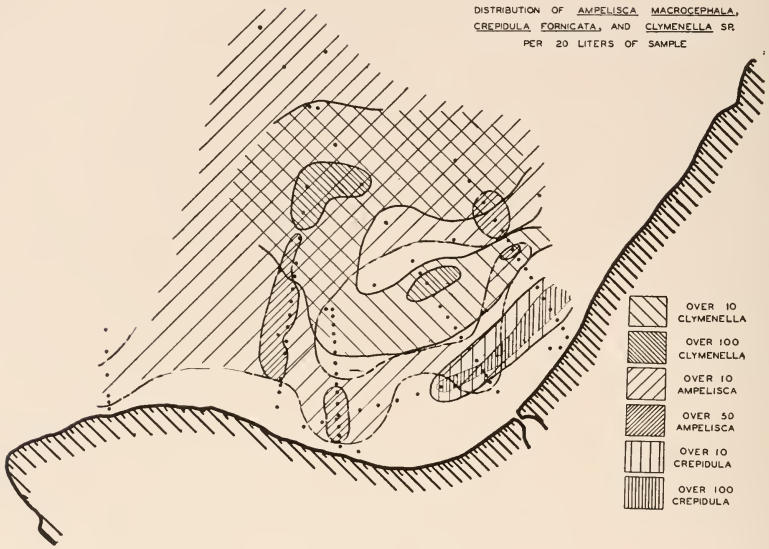

DISTRIBUTION OF AMPELISCA MACROCEPHALA, CREPIDULA FORNICATA, AND CLYMENELLA SP. PER 20 LITERS OF SAMPLE
OVER 10 CLYMENELLA
OVER 100 CLYMENELLA
OVER 10 AMPELISCA
OVER 50 AMPELISCA
OVER 10 CREPIDULA
OVER 100 CREPIDULA

CHART 4. The distribution of the three most numerous invertebrate animals in Menemsha Bight. The regions in which a common organism is most abundant are likewise characterized by a relatively greater number of different species (cf. Chart 3).

Zone 4

In the region about stations 89 and 90, a patch of medium grained white sand was discovered. There was little qualitative difference between the fauna of this zone and that found in Zone 3 (Table II). There was a slight quantitative difference, however, for the catches from Zone 3 were somewhat greater than those obtained from Zone 4 (Table III).

Zone 5

This zone was found between the 10- and the 14-meter depth contours along the eastern shoreline of the Bight (Chart 2). The bottom material of this region was of a soft fine clay, containing stones of walnut size and a considerable amount of nitrogenous matter near its surface. Mr. H. C. Stetson [5] informs me that this bottom deposit is probably of glacial origin, and contains very little marine sedi-

[5] The author is indebted to Mr. Henry Stetson, Marine Geologist at the Oceanographic Institution, for his identification of the bottom material in the major zones of Menemsha Bight.

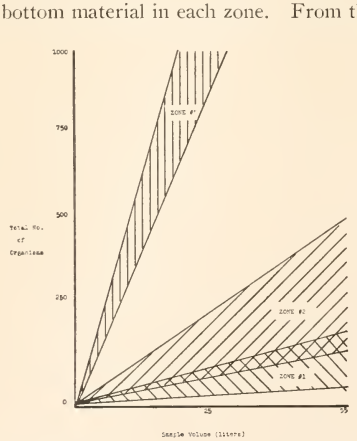
TABLE IV

The distribution of the eight most common organisms in Menemsha Bight (number of organisms/sample)

	Zone 1 15 stations	Zone 2 63 stations	Zone 3 5 stations	Zone 4 3 stations	Zone 5 5 stations
Ampelisca macrocephala	9	33	15	12	3
Clymenella torquata	0	35	1	0	0
Crepidula fornicata	0	0	0	0	423
Echinarachnius parma	3	1	4	6	0
Callocardia morruhuana	0	2	1	1	3
Cyprina islandica	0	0	4	1	0
Tellina tenera	9	0	0	0	0
Emerita talpoidia	8	0	0	0	0

ment. The bottom fauna of this region was composed of several types of crustaceans, annelids and molluscs; and the latter were predominant by number as well as by weight (Table II). *Crepidula fornicata* was exceptionally numerous and was restricted to this zone. *Trophonia affinis,* a burrowing annelid, was likewise found only in this bottom material. None of the remaining organisms was relatively abundant. The wet weight and the organic matter contained in the large numbers of Crepidula were included in the data presented in Table II although the relative importance of this organism as an article of fish diet has not yet been established. For this reason, the value for catches obtained from this zone is unusually high (Charts and Table II).

Table 4 shows the average number of the eight most common organisms obtained per 20 liters of bottom material in each zone. From these data it is possible



GRAPH 3. The total number of animals taken in each sample from the three major Zones, plotted against the volume of the sample in liters. (Fifty-five liters, the size of the largest sample, is the linear equivalent of 750 organisms.) The values for each of the Zones tend to occur within discreet regions.

to establish: 1. the numerical distribution of any one of these most abundant animals over the area; 2. the qualitative and quantitative aspects of the fauna of each zone, with respect to these organisms; and 3. the probability of capturing one or several of these organisms in a particular locality or type of bottom. The relative abundance within the zones of such organisms as Ampelisca and Clymenella and the restricted occurrence of Crepidula, Tellina and Emerita are clearly shown.

When the volume of bottom material obtained in each sample is plotted against the total number of organisms captured in that sample, it is evident that the larger samples tended to contain the greatest number of organisms (Graph 2). Graph 3 shows the relationship between the volume of the sample and the numbers of animals it contained, in the three major zones. It is interesting to note that the ratio between the size of the sample and the number of animals taken from it follows a distinctive trend in each region. The ratio ranges from 0.083 to 0.30 in Zone 1, with an average value of 0.11 (Graph 3). Similarly, the ratios for Zone 2 and 5 are 0.47 and 1.30 respectively. These figures afford a rapid means of comparing the relative abundance of organisms in each zone. In addition to their comparative value within Menemsha Bight, they offer a new method of expressing quantitative numerical relationships or comparisons between fauna of similar bottom types which may obtain in other localities. Other aspects of the bottom fauna of different regions (wet weight, content of organic matter, etc.) can likewise be compared in this fashion.

Discussion

It is apparent from a study of Charts 3 and 4 that the abundance of various species and the abundance of individuals of a single species varies considerably over the Bight. In general, the regions containing a large number of individuals of a single common species are also characterized by numerous different species. It can therefore be assumed that these regions probably offer an optimum of the conditions which determine the occurrence of these organisms. On the basis of this assumption, a closer study of these particular areas where the common animals in the Bight, such as *Crepidula fornicata* and *Clymenella torquata* are most numerous, should reveal certain of the factors which influence their abundance and distribution. Such a study is of particular importance, since many of these areas occur within that zone (2) which is by far the largest in the Bight, and which also contains the greatest numbers of animals known to be of value as food for bottom fishes.

The distribution of *Crepidula fornicata* apparently illustrates the role which the substratum may assume in determining the occurrence of an animal. This gastropod requires some relatively large hard object, such as a stone or shell, to which it fastens itself by the broad muscular foot. Such objects were found only in Zone 5, and these organisms were restricted solely to this region.

Another instance where the nature of the bottom material undoubtedly is of importance in governing the distribution of an animal, is shown by the occurrence of *Clymenella torquata* (Chart 4). This annelid is found largely in Zone 2, although a slight overlap exists to a coarser sand bottom at station 39, and to a softer bottom (which may also contain some fine sand) at stations 2, 79, 3, 4, and 5 (Charts 4 and 2). This organism constructs small tubes of the sand particles. From a comparison of the occurrence of the animal with the existence of a fine sand bottom, it

would seem that these annelids may be limited to those regions of Menemsha Bight where the bottom material contains at least a certain amount of the optimum size sand particles.

The distribution of Clymenella in the Bight also suggests that its abundance in certain regions is associated in some manner with the depth of the water. At stations 76, 77, and 21, a standard unit of bottom material contained over 100 specimens, while at station 22 they were just below this number (Chart 4). These stations border the 20-meter depth contour, which is also the outermost (deeper) boundary of the fine bottom in Zone 2. At station 11, where the depth of water increased relatively suddenly from 15 to 21 meters, the number of Clymenella is over 10 times greater than that found at adjacent stations, where the depth was 15 meters. Thus it is possible that the depth of the water, in addition to the nature of the bottom, may influence the abundance of this organism. The relative scarcity of Clymenella at stations 15, 16, and 20, however, cannot be due either to the depth of water or the nature of the bottom material. Additional factors such as prevailing tidal currents, which are at present undetermined, may also influence the occurrence of this animal.

In addition to the qualitative and quantitative variation in the fauna of different zones and of different regions of the same zone, a fluctuation was also found in the numbers and species present in the fauna of adjacent stations over a uniform bottom (Chart 3). This variation is due primarily to two causes. In the first place, many of the organisms in the Bight are widespread, but they are also sparsely distributed (Cirratulus, Cancer, Callocardia, Libinis, Polynices, Limulus, etc.). The capture of several of these animals at one location, and their absence at the next, produced a considerable fluctuation in the number of species taken in each sample. As these organisms are also relatively large, their occasional occurrence likewise produced a variation of 10 to 20 grams in the wet weight and of 1 to 2 grams in the dry weight of the organic matter contained in the catches at adjoining stations. Secondly, it has been shown that the abundance of a single common species (*Clymenella torquata*) at two adjacent stations may vary 50 to 80 per cent from an average value, when the numbers of such organisms present in the region are relatively low (Table II). This fluctuation cannot be associated with any obvious characteristics of the environment, such as the depth of the water, or the nature of the substratum. It may represent an inherent tendency of the organisms to aggregate in certain arbitrary regions of the bottom, or it may be caused by environmental factors small enough to be completely overlooked in a survey of this general nature.

The bottom fauna of Menemsha Bight was similar to that found in European flounder fishing grounds, in that molluscs, annelids, and small crustaceans were the most numerous organisms. The molluscs of the European fisheries served as the chief article of food for the bottom dwelling teleosts in the region, with the annelids and the crustaceans as the next most preferred diet (Jensen, 1919; Davis, 1923; Blegvad, 1925, 1928, 1930). However, the actual food relationships which exist between the flounder and the bottom invertebrate fauna in Menemsha Bight have not been determined at present. During two trips over the region with a commercial fishing trawler, it was found that the flounder are taken in greatest number from two widely separated belts at maximum distances of 1.0 and 3.0 kilometers from

the shore (Chart 5). It is evident that the abundance of flounder and that of Clymenella and Ampelisca (Chart 4) occur in relatively the same regions, but the actual nature of this relation remains to be established by further studies.

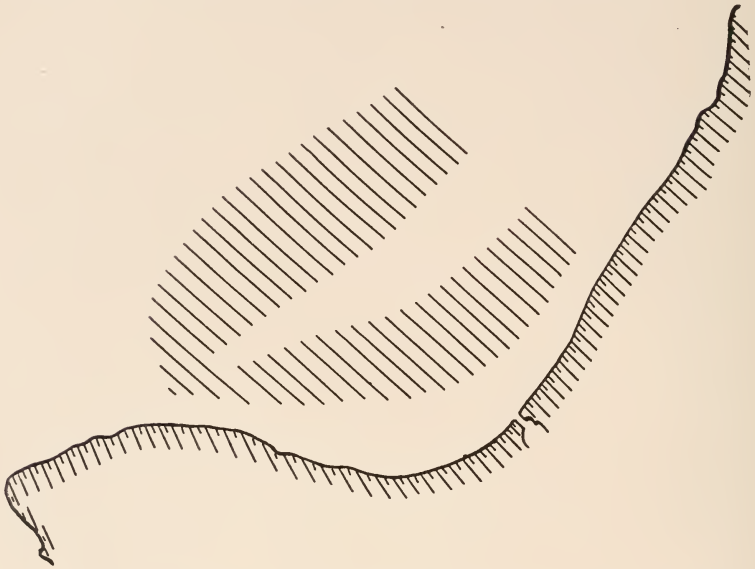


Chart 5. The diagonal lines indicate the regions of Menemsha Bight where the flounder are captured in greatest abundance. By comparing this chart with Chart 4, it is seen that these regions coincide in general with those areas where *Ampelisca macrocephala* and *Clymenella torquata* occur in large numbers.

Summary

1. A dredge which is larger and heavier than those employed previously in quantitative marine bottom sampling has been used with a specially designed screening device to facilitate the removal of organisms from the relatively large samples of bottom material.

2. The bottom of Menemsha Bight, a flounder fishing grounds in Vineyard Sound, is divisable into five zones on a basis of the bottom material. Four of the zones possessed a distinct faunal aggregation.

3. The zone which extended from the 5- to the 20-meter depth contour and contained a fine sand bottom occupied 80 per cent of the area of the Bight. This zone contained the greatest quantity of the animals (per unit volume of bottom material) that are generally known to serve as food for fishes. *Clymenella torquata* and *Ampelisca macrocephala* were the most common organisms. Flounders are caught in greatest numbers in the regions where these animals are most abundant.

4. The numerical distributions of *Clymenella torquata* and *Crepidula fornicata* are correlated with distinct characteristics of the bottom.

5. In general, the regions of the Bight containing an abundance of individuals of a common species also contain a large number of different species.

6. The fauna of adjacent stations in a relatively uniform area (Zone 2) may differ considerably in both qualitative and quantitative fashion. This variation is not associated with any observed environmental differences, such as the nature of the bottom material or the depth of water. It may represent the influence of minor factors, or of mechanical aggregation.

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AN EXPERIMENTAL STUDY OF PROTOPLASMIC pH DETERMINATION.[1] 1. AMOEBAE AND ARBACIA PUNCTULATA

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Numerous attempts have been made to determine the pH of various parts of different cells. The results obtained by these investigations are not always in agreement. The literature up to 1926 is reviewed in a monograph by Paul Reiss (1926); for reference to more recent papers see Heilbrunn (1943). The picture presented in the literature, as a whole, is not a consistent one. If the question is to be settled, further work is necessary.

The purpose of this paper is to present observations on pH determinations performed by means of the injection of indicators into amoebae and the eggs of *Arbacia punctulata*. Methods are examined carefully in order to evaluate their sources of error, and the assumptions upon which their procedures are based. Some of the contradictory values in the literature are certainly due to variations in technique and the inadequacies of methods. A preliminary note has already been published, Wiercinski (1941).

This problem was suggested by Dr. L. V. Heilbrunn and I wish to express my appreciation for his advice and criticism.

Materials, Methods, and Techniques

The organisms used included *Amoeba proteus, Amoeba dubia,* and the eggs of *Arbacia punctulata. Amoeba proteus* and *Amoeba dubia* were taken from subcultures of organisms originally obtained from Dr. J. A. Dawson. These amebae were cultivated in glass finger bowls containing about 100 cc. of a modified Ringer's solution (Hopkins, 1926), to which a boiled wheat kernel and a piece of hay stem had been added. This medium was allowed to stand for a week or two until a bacterial flora developed and then the amebae were introduced. The modified Ringer's solution was made from water distilled in a metal still. Arbacia eggs were obtained as recommended by Just (1939). A sample from each batch of eggs was fertilized. If the number of eggs showed less than 98 per cent of elevated membranes the batch was discarded.

In most cases the amebae and sea urchin eggs were centrifuged before study. Centrifugation was carried out with an Emerson electric centrifuge which developed a force of about 6000 times gravity. For the Arbacia eggs, a layer of isotonic

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sucrose solution (0.73 Molar) at the bottc of the centrifuge tubes acted as a cushion. The centrifuging process was cont..nued until the fifth layer of granules described by Harvey (1932) was plainly visible.

Clark and Lubs indicators in 0.4 per cent solution were used as noted below for injection into Arbacia eggs. Certain of the indicators were used in combination as mixed indicators. For the amebae the mixed indicators were used in a 0.2 per cent concentration. A mixed indicator has the advantage of a sharp color transformation point (pT) at a given pH (see Kolthoff and Rosenblum, 1937).

The following indicators were employed: phenol red (PR), brom thymol blue (BTB), brom cresol purple-brom thymol blue (BCP–BTB), pT at pH 6.7, brom cresol purple (BCP), brom cresol green-chlor phenol red (BCG–CPR), pT at pH 6.1, chlor phenol red (CPR), chlor phenol red-aniline blue (CPR–AB), pT at pH 5.8, 0.2 per cent methyl red (MR), brom cresol green-sodium alizarine sulfonate (BCG–SAS), pT at pH 5.6 and brom cresol green (BCG).

A comparison with microstandards was made to check the color reaction observed in the cells. The McIlvaine buffer was prepared (see Clark, 1928) and 0.25 cc. of a fresh 0.4 per cent indicator solution was added to 10 cc. of buffer. The indicator-buffer mixture was drawn into a Pyrex capillary (0.8 mm. external diameter and 0.6 mm. internal diameter), the ends sealed in a microflame and dipped in warm paraffin in order to obtain an additional seal. The microstandards were fixed on a slide about 2 mm. apart in clarite under an oblong coverslip (22 mm. × 40 mm. No. 2).

The color characteristics of the mixed indicator standards were as follows:

BCP–BTB, pT at pH 6.7. The range was taken from pH 5.7 to 7.3 at 0.2 pH intervals. At pH 7.3 the color reaction was greenish blue. The blue color diminished at pH 7.1 and 6.9. From pH 6.7 to 6.1 the color was predominantly green. At pH 5.9 the color was yellowish green and more green at pH 5.7.

BCG–CPR, pT at pH 6.1. The range was taken from pH 6.9 to 5.3 at 0.2 pH intervals. Above pH 6.1 the color increased in violet intensity. Below pH 6.1 the color appeared green and at pH 5.3 a yellowish green.

CPR–AB, pT at pH 5.8. The range was taken from pH 6.6 to 5.0 at 0.2 pH intervals. Above 5.8 the color was increasingly blue. At pH 5.8 the reaction was blue green and below pH 5.8 the color was increasingly green.

BCG–SAS, pT at pH 5.6. The range was taken from pH 6.4 to 4.8 at 0.2 pH intervals. On the alkaline side of the transformation point, this indicator exhibited a violet hue at pH 5.6 and was increasingly blue to pH 6.4. On the acid side, the color appeared to decrease in green to a yellow green at pH 4.8.

The Chambers micromanipulator was used for the microinjection of indicators into the amebae and the eggs of *Arbacia punctulata*. The material was prepared in a hanging drop over a moist chamber. Micropipettes were machine drawn from Pyrex capillary tubing (0.8 mm. external diameter) and the tip was approximately one micron in diameter when broken against the coverslip. The micropipette was washed once in the indicator solution before injections were performed. Also, by means of the Schmidtmann technique indicator particles were introduced into the amebae (see Schmidtmann, 1924; 1925).

Arbacia eggs were centrifuged in sea water and immersed in a particular medium for injection. The media used were as follows: sea water at pH 8.0 and pH 5.0;

2 parts sea water and 1 part 0.29 M. $CaCl_2$, pH at 7.6; 2 parts 0.29 M. $CaCl_2$ and 1 part sea water, pH 7.2; 70 parts sea water and 30 parts 0.53 M. NH_4Cl, pH 6.7; and 0.29 M. $CaCl_2$ at pH 6.1 and pH 7.0 (buffered with glycyl-glycine).

In most of the experiments, ten sea urchin eggs were microinjected for each medium and indicator. In all the experiments over 1000 eggs were observed. In the NH_4Cl-sea water mixtures, the eggs were allowed to remain in the solution in a shallow dish. They were then centrifuged and microinjected in the same medium.

Observations

Amoeba proteus and Amoeba dubia

The data for the uncentrifuged and centrifuged amebae are indicated in Table I.

Table I

The microinjection of pH indicators into Amoeba proteus *and* Amoeba dubia

Indicator	Number of cases	Successful injections	Color		Cytoplasmic pH	Remarks
			Indicator	in ameba		
						Uncentrifuged amebae
0.4% PR	25	15	dark red	yellow	7.0	approx. ⅕ volume or less
		0		red	7.6	approx. ½ volume
				yellow	7.0	disintegration
0.4% BCP	25	8	dark red	blue	6.6–7.0	intact amebae
				yellow	5.8	disintegration
0.2% BCG–CPR	50	4	purple	dark blue	6.6	intact amebae
				green to yellowish	6.1 5.3	disintegration
0.2% BCP–BTB	50	8	purple	blue-green	6.8–7.0	intact amebae
				yellow	5.7	disintegration
						Centrifuged amebae
0.4% PR	50	28	dark red	yellow	7.0	approx. ⅕ volume or less
		0		red	7.6	approx. ½ volume
				yellow	7.0	disintegration
0.4% BCP	25	6	dark red	blue	6.6	intact amebae nucleus, pH 7.0
				dark blue yellow	5.8	disintegration

In the uncentrifuged amebae that did not disintegrate following injection, the initial reaction showed a sphering of the animal. In about 4 minutes pseudopodia were extended and the animal again appeared normal. With 0.4 per cent BCP in cases where disintegration occurred, the intact parts retained a bluish tinge of about pH 6.6. The disintegrated coagulum was a brilliant yellow color, pH < 5.8. When the indicator was released in the medium near an ameba, the movement of the pseudopodia seemed to turn the animal away from the zone of the indicator.

In the centrifuged amebae, immediately after the injection of the indicator the animal moved rapidly, redistributed the granules into the hyaline zone, and sent out many forms of pseudopodia.

Causes for failure were ascribed to injury to the ameba, overinjection of the indicator, and the toxicity of the indicator employed. In most of the cases with 0.2 per cent BCP–BTB, merely touching the ameba with the micropipette filled with indicator solution caused a violent reaction and subsequent disintegration.

Schmidtmann injection of dry indicator particles

The amebae can tolerate the injection of particles of dry indicator such as phenol red, brom cresol purple, and brom thymol blue that are not larger than one to two micra in length, width, or thickness. Comparison was made with a drop of McIlvaine buffer, pH 6.8, in which a particle of indicator was allowed to dissolve.

Forty attempts were tried and 15 of these were considered successful with phenol red. The particles colored the protoplasm quickly and were not expelled from the ameba. A churning of the interior of the animal with much pseudopodial movement was observed. However, in many cases due to injury, the ameba would disintegrate when a particle of indicator was introduced. Phenol red showed a yellow color reaction, pH < 7.0.

In two successful cases out of 20, brom cresol purple particles showed a blue color of about pH 6.6 in the protoplasm surrounding the indicator particle.

In five successful cases out of 25 brom thymol blue particles showed a green color reaction of about pH 6.6 to 6.9 in the protoplasm surrounding the indicator particle.

The microinjection of pH indicators into amebae was undertaken because of the contradictory values reported in the literature. These values are as follows: Kite (1913), pH 6.7; Needham and Needham (1925), pH 7.6; Chambers, Pollack and Hiller (1927), pH 6.8 ± 0.1; and Spek and Chambers (1934), pH 7.3. The results reported here indicate a pH of approximately 6.8.

Arbacia punctulata

Pandit and Chambers (1932) microinjected indicators into the uncentrifuged Arbacia egg in sea water. They reported a value of pH 6.8 ± 0.2 for the cytoplasm of this egg. Whenever vital dyes or indicators are used distinction must be made between the granular and hyaline portions of the cell. Pandit and Chambers did not separate the cytoplasmic components.

In the following experiments centrifuged Arbacia eggs were immersed in media of various pH and microinjected with sulfonphthalein indicator dyes. In no case was fertilization obtained after injection. The experiments were carried out at room temperature that varied from 19° C. to 24° C. In some of the injections membranes were elevated; the cortex broke down in all cases with the associated wave of reaction; the eggs appeared to swell and fifth layer granules were observed to break down with the formation of small vacuoles. In no case was a color change observed in the fat cap region. In many of the injections, the indicators seemed to be immiscible with the hyaloplasm and as a consequence membranes were formed about the droplet of indicator solution. In cases when membranes are formed the hyaloplasm may remain colorless and the color will appear as a flash. Large injections of indicator (approximately ½ volume of the egg) showed membrane formation. A smaller injection (approximately ⅕ volume of the egg or less) seemed to diffuse evenly throughout the hyaline zone.

Table II

Colorimetric pH determination of centrifuged Arbacia eggs in normal sea water, pH 8.0

Indicator	Number of eggs	Observed color reactions											Remarks
		Indicator		Membrane formation			Hyaline zone			SPR			
		pipette	sea water	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
PR	15	red	red	3	yellow	<7.0		yellow	<7.0				protoplasmic coagulum changed to red color
BTB	10	dark green	blue	8	yellow	6.4				1	none		
										1	green	6.8	
												6.6	
BCP–BTB, pT at pH 6.7	11	dark blue	blue	8	blue flash	6.9	2	blue	<6.7				fifth layer of granules green, pH <6.7; injected eggs turn blue
								green	6.0				
								yellow	<5.7				
BCP	10	red	blue	6	green	6.4				4	yellow	6.0	granular breakdown showed yellow color reaction
					yellow	6.0						5.8	
						5.8							
BCG–CPR, pT at pH 6.1	10	dark purple	blue	2	violet	>6.1	4	violet	>6.1	2	green	6.1	colors change to blue
							2	green	<6.1			5.7	
CPR	10	blue	dark blue	6	none		4	violet	6.6				
								green	6.0				
								yellow	5.0				
CPR–AB, pT at pH 5.8	10	purple	dark blue	6	blue flash	>5.8	4	none					color change observed following breakdown
					green	<5.8							
MR	10	yellow	yellow	4	yellow flash	>5.8	6	none					faint yellow difficult to observe
BCG–SAS, pT at pH 5.6	10	purple	dark blue	2	blue	6.2	8	blue	6.2				granular area green, pH <5.6; eggs become purple
						>5.6							
BCG	10	blue		3	blue	>5.4	2	none					eggs become purple
							5	blue	>5.4				

Surface precipitation reaction (SPR) membranes were seen to occur more often in the 0.29 M calcium chloride solutions than in the injections performed in sea water. In most cases following the breakdown of the egg, the protoplasmic coagulum changed to the pH of the surrounding medium. This was observed by the change in color of the injected indicator.

Normal sea water, pH 8.0

In this series 106 centrifuged eggs were injected. The results are summarized in Table II. The following results were obtained: PR, pH < 7.0; BTB, pH 6.8 to 6.4; BCP–BTB, pH < 6.7 to 6.0; BCP, pH 6.4 to 5.8; BCG–CPR, pH approximately 6.1; CPR, pH 6.6 to 6.0; CPR–AB, pH > 5.8; MR, pH > 5.8; BCG–SAS, pH > 5.6 to 6.2; and BCG, pH > 5.4. The probable pH as indicated in these results is in the range from pH 6.4 to 5.8.

Acid sea water at pH 5.0

This medium was adjusted with HCl and brought to equilibrium by bubbling air through the solution for 24 hours. The eggs centrifuged in normal sea water and then immersed in acid sea water often became sticky and distorted in shape. The eggs tended to clump together and often clogged the micropipette.

In this series 30 eggs were microinjected. The results are summarized in Table III. The results are as follows: PR, pH < 7.0; BCP, pH < 7.0 to 6.2; and MR, pH > 5.8.

Sea water with excess calcium

Two parts sea water and 1 part 0.29 M. $CaCl_2$, pH at 7.6

Ten eggs were microinjected with BTB. The results summarized in Table III indicate a pH of 6.0.

One part sea water and 2 parts 0.29 M. $CaCl_2$, pH at 7.2

Ten eggs were microinjected with BCG–BTB, pT at pH 6.1. The results summarized in Table III indicate a pH in the neighborhood of 6.1 to 5.9.

Calcium solutions

0.29 M. $CaCl_2$, buffered with glycyl-glycine to pH 7.0

In this series 80 eggs were injected and the results are summarized in Table IV. The results are: PR, pH < 7.0; BTB, pH 6.8 to 6.2; BCP, pH 6.4 to 6.0; BCG–CPR, pH approximately 6.1; CPR, pH 6.6 to 6.0; MR, pH > 5.8; BCG–SAS, pH > 5.6; BCG, pH > 5.4. The probable pH as indicated in these results is in the range from pH 6.4 to 6.0.

0.29 M. $CaCl_2$, pH at 6.1

Eggs immersed in this solution and returned to sea water after 35 minutes showed 100 per cent fertilization membranes when sperm suspension was added.

Table III

Colorimetric pH determination of centrifuged Arbacia eggs in various media

Medium	Indicator	Number of eggs	Observed color reactions											Remarks
			Indicator		Membrane formation			Hyaline zone			SPR			
			pipette	medium	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
Sea water pH 5.0	PR	10	red	red flash	1	yellow	<7.0	9	yellow	<7.0				granules showed a brilliant yellow
	BCP	10	dark red	purple flash	3	blue	<7.0 6.2	6	blue	<6.8 6.2				eggs turned yellow, pH 5.8
	MR	10	yellow	yellow	1 2	yellow none	<5.8	7	yellow	>5.8	1	yellow	5.8	yellow color spread through eggs as breakdown occurred
2 parts sea water, 1 part $CaCl_2$, pH 7.6	BTB	10	dark green	dark green	5	yellow	6.0	5	yellow	6.0		yellow	6.0	
1 part sea water, 2 parts $CaCl_2$, pH 7.2	BCG–CPR, pT at pH 6.1	10	dark purple	blue	2	violet green	>6.1 <6.1	2	violet green	>6.1 5.9	6	green	5.9	interior of SPR eggs violet, pH 6.1–6.3; all eggs turned to green in 30 to 50 seconds

Table IV

Colorimetric pH determination of centrifuged Arbacia eggs in 0.29 M. $CaCl_2$, buffered with glycyl-glycine to pH 7.0

Indicator	Number of eggs	Observed color reactions											Remarks
		Indicator		Membrane formation			Hyaline zone			SPR			
		pipette	0.29 M. $CaCl_2$, pH 7.0	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
PR	10	dark red	orange	3	yellow	<7.0	6	yellow	<7.0	1	yellow	<7.0	
BTB	10	dark green	green	1	none		9	green	<6.8				eggs turned purple-green
								yellow	<6.2				
BCP	10	red	blue	2	green	6.4		none			yellow	5.6	
						6.0						5.4	eggs turned to yellow in 30 to 60 seconds
							2	green	6.4				
									6.0				
										4	green	6.4	
												6.0	
										2	yellow	5.6	
												5.4	
BCG–CPR, pT at pH 6.1	10	dark purple	blue	9	violet	6.1				1	violet	>6.1	in 2 minutes all eggs green
											green	<6.1	
CPR	10	dark red	red to blue	3	violet	6.6	6	violet	>6.0	1	yellow	<5.6	turned yellow, pH 5.6
						6.0							
MR	10	yellow	yellow	5	yellow	>5.8	2	yellow	>5.8	3	yellow	>5.8	eggs turn to green
BCG–SAS, pT at pH 5.6	10	dark green	green	4	blue	>5.6				6	blue	>5.6	
BCG	10	dark green	dark flash	5	blue	>5.4				5	blue	>5.4	eggs turn purple, pH 5.4

TABLE V

Colorimetric pH determination of centrifuged Arbacia eggs in 0.29 M. $CaCl_2$, pH 6.1

Indicator	Number of eggs	Observed color reactions											Remarks
		Indicator		Membrane formation			Hyaline zone			SPR			
		pipette	0.29 M. $CaCl_2$, pH 6.1	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
PR	10	red	yellow flash	2	yellow flash	<7.0	4	yellow	<7.0	3	yellow	<7.0	
				1	yellow	<7.0					none		
BTB	13	red	green	3	yellow to green	6.2 6.0	3	yellow to green	6.2 6.0				micropipette yellow from adhering coagulum and indicator
							7	yellow to green			yellow	6.0	
BCP–BTB, pT at pH 6.7	20	blue	green	12	green	<6.7 6.0	7	green	<6.7 6.0				color most pronounced in region of puncture
				1	yellow	<5.8							
BCP	10	red	green				5	green	6.2 6.0	5	green	6.2 6.0	injected eggs turn to yellow, pH 5.8
											yellow	5.8	
BCG–CPR, pT at pH 6.1	10	dark purple	blue				6 4	violet violet	>6.1 <6.1		green	5.8	granules were yellow, pH 5.4
CPR	10	red	violet	3	violet	6.0				6	yellow	<5.6	appeared dark blue, pH >7.0
				1	blue to violet	6.2		violet	6.0				
CPR–AB, pT at pH 5.8	20	blue	blue	19	blue	>5.8				1	blue green	>5.8 <5.8	
MR	10	yellow	yellow flash	6	yellow	>5.8				4	yellow	>5.8	
BCG–SAS, pT at pH 5.6	10	dark blue	blue				10	blue	6.2 6.0				eggs absorbed indicator, blue, pH 6.0–6.2
BCG	10	dark green	blue				6 4	blue blue	5.6 5.6		green	5.6	change to yellow, pH 4.0

Table VI

Colorimetric pH determination of centrifuged Arbacia eggs in 70 parts sea water, 30 parts 0.53 M. NH_4Cl, pH 6.6

Indicator	Number of eggs	Observed color reactions											Remarks
		Indicator		Membrane formation			Hyaline zone			SPR			
		pipette	sea water, NH_4Cl, pH 6.6	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
PR	10	red	yellow flash	2	none		6	yellow	<7.0	2	yellow	<7.0	yellow ring of color circumscribed egg
BCP	10	red	blue	2	yellow	5.8	4	green	6.0				granular area purple
				3	green	6.4	1	blue	6.8				
					yellow	5.8		green	6.4				
								yellow	5.8				
BTB	10	red	green				9	green	6.8	1	yellow	<6.4	
									6.4				
BCP–BTB, pT at pH 6.7	10	dark blue	green ?	2	green	<6.7	5	green	<6.7				
							3	green	<6.7				
								yellow	<5.8				
CPR	10	dark red	dark blue ?	5	blue	6.6	5	blue	6.6				
						6.0			6.0				
BCG–CPR, pT at pH 6.1	10	purple	dark	3	blue	>6.1							
			blue	7	blue flash	6.1							
CPR–AB, pT at pH 5.8	12	purple	dark blue	5	blue	>5.8	7	blue	5.8				
MR	10	yellow	yellow flash	2	yellow	6.0	8	yellow	6.0				
						5.8			5.8				
BCG–SAS, pT at pH 5.6	10	green	light green				8	blue	6.0	2	blue	6.0	some eggs absorbed indicator, turned green, pH <5.6
BCG	10	dark purple	blue	4	dark blue	>5.4	6	dark blue	>5.4				

The eggs seemed to withstand much handling and manipulation. However, some batches of eggs showed a tendency to stick together when immersed in pure calcium solution.

In this series 133 eggs were microinjected and the results are summarized in Table V. The results are: PR, pH < 7.0; BTB, pH 6.2 to 6.0; BCP–BTB, pH < 6.7 to 6.0; BCP, pH 6.2 to 6.0; BCG–CPR, pH approximately 6.1; CPR, pH > 5.6 to 6.2; CPR–AB, pH > 5.8; MR, pH > 5.8; BCG–SAS, pH about 6.0 to 6.2; and BCG, pH > 5.6.

Ammonium chloride-sea water

Seventy parts sea water, 30 parts 0.53 M. NH_4Cl, pH at 6.6

Different batches of eggs were allowed to remain in this medium for about 6 hours. An increase in the size of the fat cap occurred, and close to 98 per cent fertilization was obtained. According to Jacobs (1922) ammonium chloride is believed to cause an alkalinization of the protoplasm.

In this series 112 eggs were microinjected and the results are summarized in Table VI. No color change was observed in the fat cap region. The results are as follows: PR, pH < 7.0; BCP, pH 6.8 to 5.8; BTB, pH 6.8 to 6.4; BCP–BTB, pH < 6.7; CPR, pH 6.6 to 6.0; BCG–CPR, pH > 6.1; CPR–AB, pH > 5.8; MR, pH 5.8 to 6.0; BCG–SAS, pH 6.0; and BCG, pH > 5.4. The probable pH as indicated in these results is in the range from pH 6.6 to 6.0.

Protein-lipid error of indicators

The presence of proteins and lipids is a well recognized source of error in the colorimetric determination of pH. It was of interest to test the interference of protoplasmic and lipid substances of Arbacia eggs upon pH indicators. This was done in the following manner:

The hyaline and granular halves of Arbacia eggs were obtained by means of high speed centrifugation. The respective halves were removed from the centrifuge tube with a pipette and put into separate test tubes. The portions were washed by shaking in 2 cc. of 0.35 M. sodium citrate-citric acid buffer at the desired pH. This mixture was centrifuged at low speed and the supernatant fluid was decanted. Two cc. of fresh buffer was added. The proportion was approximately $1/16$ cc. of egg substance (hyaline or granular halves) to 2 cc. of buffer. The hyaline or granular buffer mixture was transferred to a small mortar and ground with a pestle. The ground mixture was tested electrometrically with a Beckman pH meter and colorimetrically with indicators. The colorimetric determination of the ground hyaline-buffer mixture was made on a white spot plate. The proportion was $1/16$ cc. of 0.02 per cent indicator to 1 cc. of mixture. The granular halves were too dark with pigment to determine a colorimetric error.

The results are shown in Table VII. Phenol red showed a colorimetric error of pH -0.1 to $+0.02$, brom cresol purple ranged from pH -0.17 to $+0.22$, and brom thymol blue pH $+0.28$ to $+0.92$.

Colorimetric pH error was also determined with dried Arbacia substance. This was added to a 0.1 N HCl solution diluted with distilled water to various pH intervals. The proportion was 5 cc. of acid and 0.05 g. of Arbacia substance.

TABLE VII

Protein-lipid error of pH indicators determined with hyaline Arbacia substance (Na Citrate-Citric Acid Buffer)

Indicator	Electrometric pH	Colorimetric pH	Electro-Color pH
PR	7.02	7.0	+0.02
PR	7.19	7.2	−0.01
PR	7.30	7.3	0.00
BTB	6.48	6.2	+0.28
BTB	6.67	6.1	+0.57
BTB	6.83	6.1	+0.73
BTB	7.02	6.1	+0.92
BTB	7.19	6.5	+0.69
BTB	7.39	6.7	+0.69
BCP	5.64	5.5	+0.14
BCP	5.82	5.6	+0.22
BCP	6.04	6.0	+0.04
BCP	6.18	6.1	+0.08
BCP	6.48	6.1	+0.08
BCP	6.67	6.5	+0.17
BCP	6.83	7.0	−0.17

The results are shown in Table VIII. Phenol red showed a colorimetric error of approximately pH + 0.3, brom thymol blue approximately pH + 0.1 to + 0.4, and chlor phenol red approximately pH + 0.2 to + 0.4.

TABLE VIII

Protein-lipid error of pH indicators determined with dried Arbacia substance (0.1 N HCl Solution)

Indicator	I Electrometric pH	II Colorimetric pH	I–II Electro-Color pH
PR	7.30	7.0	+0.3
BTB	6.43	6.3	+0.13
BTB	6.80	6.4	+0.4
CPR	6.12	5.7	+0.42
CPR	5.78	5.5	+0.28
CPR	5.72	5.5	+0.22

DISCUSSION

Chambers (1929) and his coworkers have injected pH indicators into many types of cells. They arrive at a value of pH 6.8 ± 0.2 for the cytoplasm of all of them. If this is correct, it is a most interesting generalization.

In this country and in England, the data presented by Chambers have been universally accepted. However, in the European literature other pH values are reported as follows: Reiss (1928) cytoplasm of the *Paracentrotus lividus* egg, pH 5.6, Vles and Vellinger (1928) cytoplasm near the pigment granules in Arbacia, pH 5.5 ± 0.3, Spek and Chambers (1934) hyaloplasm of *Amoeba dubia,* pH 7.3,

Reiss and Gersch (1936) macromeres and micromeres of *Aplysia limacina* somewhere between pH 6.0 and 7.0, and Raven (1937) cytoplasmic inclusions of Chaetopterus, Nereis, and Aplysia eggs are acid and basic, and the hyaline zone is not colored by indicators.

Work with amebae indicated that these organisms were able to tolerate the microinjection of minute quantities of sulfonphthalein indicator dyes (see Reznikoff and Pollack, 1928). Phenol red was the least toxic of all the indicators used. In *Arbacia punctulata* eggs, the microinjection of sulfonphthalein indicator dyes was followed by a wave of reaction in which a breakdown of cortical and fifth layer granules occurred. In most of the cases the indicator was found to be immiscible with the hyaloplasm as seen in the numerous instances of membrane formation about the zone of injection.

The results show that the protoplasm of different types of cells does not have the same pH but that, for the cells examined, the pH lies within a range from pH 6.0 to 7.0. Ameba hyaloplasm appeared to be more alkaline than the hyaloplasm of sea urchin eggs.

In all the media, the observed colorimetric pH values for the Arbacia hyaloplasm were in the range from pH 5.8 to 6.8. In agreement with Chambers (1929) for other eggs, the nucleus was found to have a pH above 7.0. After breakdown of the fifth layer of granules, the pH of the granular material was found to be 5.4. Upon complete cytolysis, the cytoplasmic pH becomes more acid. After the external medium invades the cytolyzed egg, the internal pH is similar to the external pH. Injections made in the heavy granule area gave inconsistent and uncertain results.

The pH of the surrounding medium does not appear to have much effect on the hyaloplasm pH (also see Chambers, 1928). The pH value for individual measurements showed some variation but this was not related to the pH of the surrounding medium. Thus, when Arbacia eggs were immersed in sea water at pH 8.0 as compared with sea water at pH 5.0, no marked differences were observable in the initial pH reading. Moreover, when eggs were immersed in sea water containing NH_4Cl under conditions in which alkalinization of the protoplasm is believed to occur, no such effect could be observed in the hyaloplasm. In the original observations of Jacobs (1922) of alkalinization in such a solution, the observations were made with uncentrifuged eggs in neutral red solution which stains the granules. Chambers (1928) discusses the granular pH in this regard. Presumably, the granular pH, that is to say the pH within the vacuoles of the granules, is affected by the ammonium ion. See Harris (1939) for a description of granular vacuoles in Arbacia eggs.

Errors of visual color determination in the microscopic field are due to the "personal equation" of the observer, rapidity of the color change, and light conditions. Colors can be altered by the reflected colors of their surroundings, by the character of light in which they are observed, the condition of rest or fatigue of the eye, and the presence of after image on the retina (see Maerz and Paul, 1930).

Inasmuch as cells contain salt and protein plus lipid, there is a possibility of salt and protein error. However, observations indicate that these apparently are not great except in the case of protein-error of Arbacia substance for brom thymol blue as indicated in the results.

In this work, distinction between granules and hyaloplasm has been attempted by centrifuging. This has not been done before in the colorimetric study of protoplasmic pH determination. The factor of indicator volume to cell volume in the quantitative study of pH with the colorimetric method is important. A method has not yet been worked out where it is possible to inject the same amount of indicator into several successive cells. This procedure is necessary to obtain more than approximate values.

The toxicity of indicators and the chemical make-up of standard indicator solutions must be considered. A 0.1 N NaOH solution is the basic solution in which most sulfonphthalein indicators are dissolved. Seemingly, the introduction of an alkaline solution into the cell would give more alkaline pH values, especially when the indicator solution is approximately $\frac{1}{5}$ to $\frac{1}{2}$ of the cell volume.

Conclusions

Within the limitations of the methods used the following values are believed to represent the approximate protoplasmic pH for the various cells studied:

Amoeba proteus pH 6.8 ± 0.2
Amoeba dubia pH 6.8 ± 0.2
Arbacia punctulata eggs pH 6.2 ± 0.2

Protein-lipid errors of indicators determined with Arbacia substance vary from pH -0.17 to approximately $+0.9$ for various indicators.

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THE INDUCTION OF LARVAL MOLTS IN DROSOPHILA

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It has recently been shown (Bodenstein, 1943) that a hormone produced by the ring gland controls the growth of the imaginal organ anlagen in Drosophila during the larval period, as well as the growth of purely larval organs. Since molting is mainly a process by means of which the larval insect grows, one might expect it to be governed by the same principle, yet previous experiments were unable to shed any light on this point. It is the purpose of this investigation to provide convincing evidence that the molting of Drosophila larvae is under the control of the larval ring gland.

Material and Methods

The larvae of Drosophila molt twice before they pupate. At each molt they shed their mouth parts, together with their cuticula. The mandibular hooks of the mouth armature, characteristic in size and shape for each larval instar, were used as a criterion for molting. For the experiments the larval head segments, including the complete mouth armature, were cut off from larvae shortly after their emergence from the egg and from 2nd instar larvae. Special care was taken not to include the brain and attached ring gland in the isolated head segments. The brainless head pieces were then transplanted into the body cavity of adult flies or into mature larvae. The transplants, either alone or together, were put with several ring glands into the body cavity of the adult hosts. The hosts were dissected at different times after the operation; the transplants removed *in toto,* stained with orcein, cleared and mounted in diaphane. The number of mandibular hooks present in the transplant could easily be observed in these preparations. In many cases one could also use the spiracles as an indicator for molting, because these structures are shed at each molt and are characteristic for each instar. The majority of experiments was performed on *Drosophila virilis* (wild stock). The experimental animals were kept at a constant temperature of $25° \pm 0.5°$ C.

I am greatly indebted to Dr. L. C. Dunn and Dr. T. Dobzhansky for many stimulating discussions and for their continued interest in the work.

Experiments

The transplantation of larval head segments into adult male hosts

In a series of experiments, larval head segments without brain and ring gland, but containing the complete mouth apparatus, were transplanted into the abdomen of adult male flies and dissected at different times after the operation. These experiments comprise three different experimental groups. In one group the head

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segments of 1st instar larvae not older than 4 hours were used. In the second group the head segments of the larvae were somewhat older but their age never exceeded 10 hours. In the third group head segments of 2nd instar larvae were transplanted. The experiments are summarized in Table I. They show that none of the transplanted head parts has molted, since only one pair of mandibular hooks is present. Usually there are some brownish spots in the grafts, while the rest of

TABLE I

Transplantation of larval head segments into the abdomen of adult virilis male flies

Age of transplanted head segment	Number of cases	Days transplant remains in host	Number of cases where transplant has only one pair of mandibles, i.e. has not molted
1st instar (4–6 hrs. old)	1	4	1
	4	7	4
	3	8	3
	4	11	4
	10	14	10
	1	15	1
	13	16	13
	3	17	3
	7	18	7
	3	19	3
1st instar (ca. 10 hrs. old)	4	16	4
2nd instar	12	8	12
Total number of cases	65		65

the tissue maintains its larval color characteristics. Grafts which remained very long in the host frequently show some brownish cuticle coloration, which resembles the color of young pupae. Whether this color is an indication of pupation is, however, questionable. Strong muscle contractions of the grafts at dissection demonstrate very well their living condition.

The transplants undergo certain other changes while in their adult hosts. It is often noticed that the cuticle between two adjacent segments becomes somewhat darkened in transplants left for 2 or 3 days in their hosts (Fig. 1). If left longer in the host the larval head pieces exhibit a typical behavior. A fine epithelial membrane begins to appear on the proximal end of the transplant where the body wall of the larvae was cut. This epithelium grows gradually, creeping around the outside of the larval cuticle of the head part until finally the whole transplant is enveloped by a fine transparent tissue sac. Figures 2, 3, 5 and 6 show this condition for some selected cases. It should be explained however that the completeness of this overgrowth does not entirely depend upon the time the implant remains in the host, for in some cases the overgrowth might have been enveloped only one-half, in others the whole of the transplant, although all remained for the

same length of time in the host. The tissue envelope apparently represents the regenerating wound epithelium of the larval epidermis. Its cells are very large and extremely flat, with large nuclei containing polytene chromosomes. In life the space between the epithelium mantle and the larval cuticula appears transparent and empty. In sectioned material, however, it seems to be filled with a laminated deposition (Fig. 6). Whether this deposit is chitin has not been determined.

Piepho (1938) in his studies on Galleria (wax moth) has observed the same type of behavior. He transplanted small pieces of caterpillar skin into the abdomens of other caterpillar hosts, and found that an epidermal cover (*Umwachsungshypodermis*), originating from the epidermis of the transplant, gradually enveloped the implant. In a careful histological study, Piepho found, moreover, that the differentiation achievements of this enveloping tissue sheath may vary according to the hormonal situation of the host.

The transplantation of larval head segments together with ring glands into the abdomen of adult flies

Head segments of 1st and 2nd instar larvae without brain and ring gland were transplanted into the abdomen of adult flies. Unlike the former series, however,

TABLE II

Transplantation of head segments together with ring glands into the abdomen of adult flies

Age of transplanted head segment	Host and transplanted ring gland	Number of cases	Days transplant remains in host	Number of cases where transplant has formed				
				1 pair of mandibles	2 pairs of mandibles	3 pairs of mandibles	spiracle of 2nd inst.	spiracle of 3rd inst.
1st instar (4 hrs. old)	v. 2RG	4	8	3	1			
1st instar (4 hrs. old)	v. 2RG	2	15	1	1			
1st instar (4 hrs. old)	v. 2RG	6	16	4	2?			
1st instar (4 hrs. old)	v. 2RG	1	5	1				
1st instar (4 hrs. old)	v. 2RG	2	7	2				
1st instar (6 hrs. old)	m. 2RG	3	7		2	(1?)		1
1st instar (10 hrs. old)	m. 2RG	9	15	2(4?)	2	(1?)	3	(1?)
2nd instar	v. 3RG	6	8	4	2			3(1?)
2nd instar	m. 3RG	3	6	2	1			
2nd instar	m. 3RG	3	8	2	1			3
Total number of cases		39			14 positive			

v. = virilis; m. = melanogaster.
RG = Ring gland.
Number in parentheses indicates number of cases where the mandible or spiracle number formed is not clear.

two or three ring glands from mature larvae were transplanted simultaneously with the larval heads into the same hosts. Not only adult males but also adult virilis females were used as hosts. In many cases adult melanogaster females were employed as hosts, for it had been found previously (Bodenstein, 1943) that the ring gland effect in melanogaster females was much stronger than in virilis females. The results of these experiments are summarized in Table II. They show that in the presence of ring glands the larval head segments can be induced to molt, as indicated by the presence of two pairs of mandible hooks in one transplant. Figures 7 and 10 show a 1st instar transplant and Figure 8 shows one 2nd instar transplant after molting has been induced by the grafted ring gland. In only one case (one other questionable), molting had occurred twice. Although only two pairs of mandibles were found in this case, one pair belonging to the 1st and the other to the 2nd instar, a spiracle typical for the 3rd instar was present, proving that the second molt had taken place (Fig. 11). As far as the molting competence of mandibles and spiracles is concerned, it seems that the spiracles react somewhat more readily to the molting hormone of the ring gland than the mandibles. This is indicated by the fact that of six 2nd instar transplants only three possessed two pairs of mandibles, while all six heads had formed 3rd instar spiracles.

Apart from their molting, the developmental behavior of these transplants is very similar to that discussed in the foregoing section. They apparently form the same type of regenerated wound epidermis cover which envelops either part or the whole of the head segments. The cuticular color of the graft under the epidermal

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PLATE I

FIGURE 1. Head segments of 2nd instar larva transplanted into an adult virilis male host 8 days after the operation. Note darkening of segment borders.

FIGURE 2. Head segments of 1st instar larva transplanted together with two larval ring glands into an adult virilis male host. Transplant remained 15 days in host but has not molted. Note that an epidermal sheath has enveloped about one-half of the transplant.

FIGURE 3. Head segments of 1st instar larva transplanted into an adult virilis male host 14 days after the operation. Note that an epidermis sheath has completely surrounded the transplant.

FIGURE 4. Head segments of 1st instar larva transplanted into an adult virilis male host 14 days after the operation. An epidermis sheath has almost completely overgrown the transplant, leaving only the tip of the head free. At the proximal end of the transplant note the two quite extensively developed eye discs.

FIGURE 5. Section through head segments of a 1st instar larva, transplanted into an adult virilis male host 17 days after the operation. The epidermis has surrounded only one-half of the transplant. Note the large nuclei(n) in the epidermis cover.

FIGURE 6. Section through head segments of a 1st instar larva transplanted into an adult virilis male host 18 days after the operation. The epidermal envelope covers the whole transplant. Note the large nuclei(n) of the epidermal envelope and also the striated material between the transplant and the epidermal sheath.

FIGURE 7. Head segments of 1st instar larva transplanted into mature larvae 5 days after the operation. Three pairs of mandibular hooks, indicating that the transplant has molted twice.

FIGURE 8. Head segments of 2nd instar larva transplanted into a mature larva 8 days after the operation. The transplant has molted but once, as indicated by the presence of only two pairs of mandibular hooks.

FIGURE 9. Head segments of 1st instar virilis larva transplanted into a mature pseudoobscura larva 7 days after the operation. Note three pairs of mandibular hooks, the third and largest pair somewhat proximal to the others. The transplant has thus molted twice.

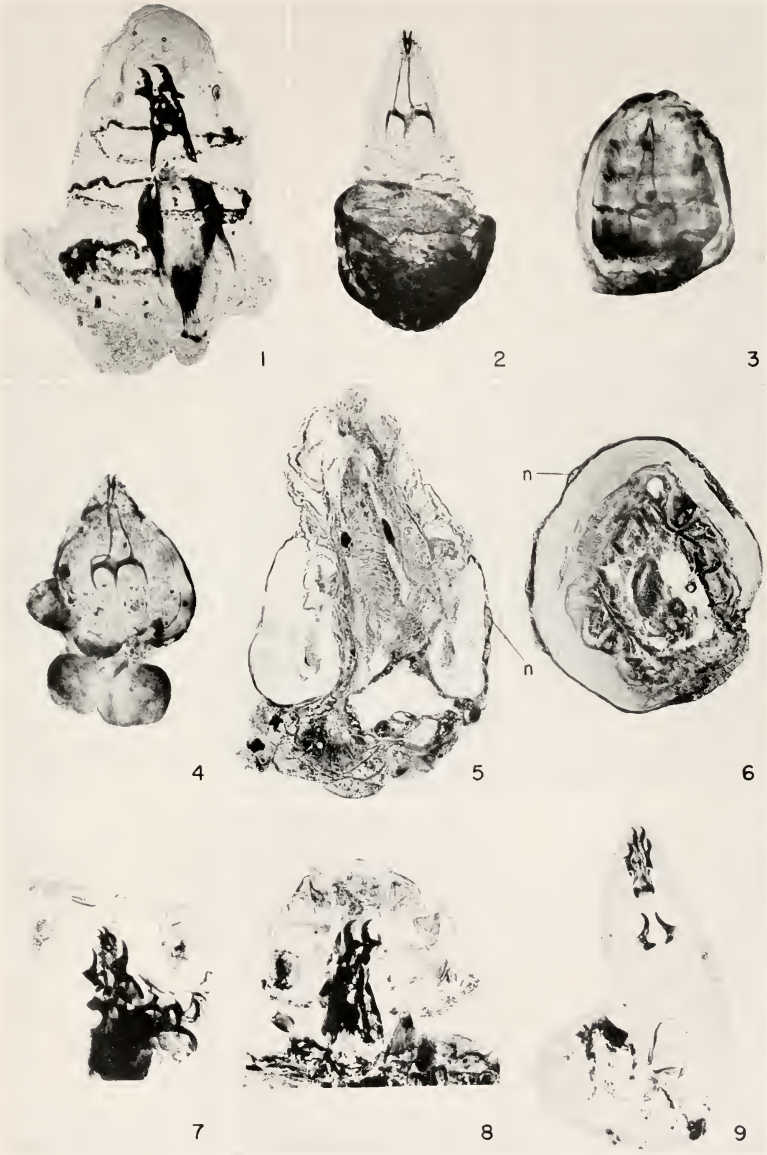

PLATE I

envelope also varies widely, from strictly larval color to darkish spotting or complete browning of the cuticle. Whether or not the browning of the cuticle is an indication of pupation remains questionable. A detailed histological study of the structure of the head parts has not been made. Thus too much emphasis cannot be placed on their apparently similar appearance.

It is interesting that the environment most favorable for molting is apparently that of melanogaster females. Three 1st instar head parts (Table II) which remained in melanogaster females for 7 days molted, while of six transplants which remained in virilis males or in virilis females for 7 to 8 days, only one molted (see Table II).

TABLE III

Transplantation of larval head segments into larval hosts

Age of transplanted larval head segments	Days transplant remains in host	Number of cases	Number of cases where graft has formed		
			1 pair of mandibles	2 pairs of mandibles	3 pairs of mandibles
1st instar (4 hrs. old)	5	6		4(1?)	1
1st instar (4 hrs. old)	6	1			1
2nd instar[a]	2	1	1		
2nd instar[a]	5	3	3		
2nd instar[a]	6	7		1(6?)	
2nd instar[a]	7	3		3	
2nd instar	5	1		1	
2nd instar	6	1		1	
2nd instar	7	6		6	
	Heteroplastic (virilis into pseudoobscura)				
1st instar (0–2 hrs. old)	5	1		1	
1st instar (0–2 hrs. old)	7	5			5
Total number of cases		35			24 positive

[a] Head skin removed, only mouth armature transplanted.

The transplantation of larval head segments into the abdomen of larval hosts

The object of this group of experiments was to test the molting behavior of larval head segments in a larval and pupal environment. For this the brain and ring gland were removed from head segments of 1st and 2nd instar larvae, which were then transplanted into the abdomen of 3rd instar host larvae. The transplanted pieces were dissected shortly before or after the emergence of the fly. Since the pupal life of virilis is about 5 to 5½ days, the head segments dissected 6 or 7

days after the operation were originally transplanted into larvae younger than those which were dissected 5 days after the operation. The head segments in these later dissected hosts therefore had remained longer in a larval environment. A few of the transplants were dissected from younger pupae. The results of these experiments are summarized in Table III, where it can be seen that molting occurred in the majority of the cases. Figure 8 shows such a case. All the head segments in this experimental combination reveal a dark brown cuticle which indeed resembles a pupal cuticle. Unfortunately, however, no special effort was made to determine

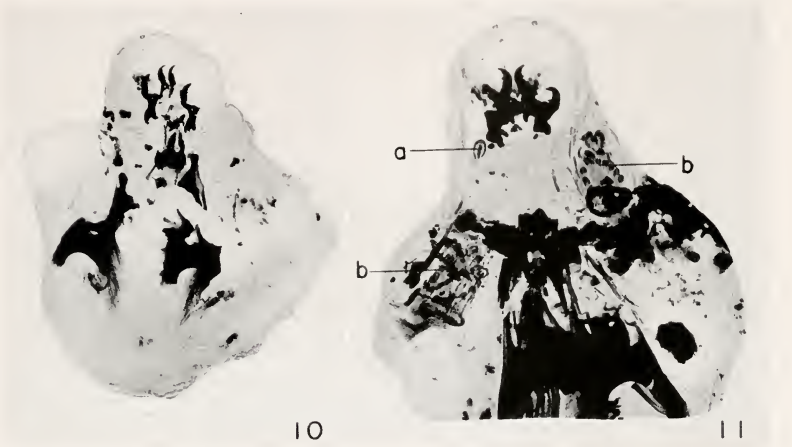

FIGURE 10. Head segments of 1st instar larva transplanted together with two larval ring glands into the abdomen of an adult melanogaster female; 7 days after the operation. The transplant has molted once. Note the two pairs of mandibular hooks.

FIGURE 11. Head segments of 1st instar larva transplanted together with three larval ring glands into an adult melanogaster female 8 days after the operation. The transplant has molted twice. Note the two pairs of mandibular hooks and the spiracles of the 2nd instar (a) and of the 3rd instar (b).

whether or not any epidermal overgrowth surrounded the transplant. Some of the 2nd instar head segments were transplanted after the skin around the mouth parts was removed, with the exception of a very small piece of skin left near the distal end. Such naked mouth parts are also able to molt (see Table III).

The molting factor is not species-specific. This is demonstrated by the transplantation of head segments of 1st instar virilis larvae without brain and ring gland into 3rd instar pseudoobscura larvae. From six cases available in this series, five had formed three pairs of mandibular hooks, revealing that they had molted twice (Fig. 9). One transplant, however, dissected somewhat earlier, had molted only once (see Table III).

The growth of young organ discs in adult hosts

Some of the organ discs such as eye, legs and antennae for example, may be left behind in the transplant after the removal of the nervous system, for these organ anlagen are too minute in size to be recognized in the dissection of the 1st instar larval transplants. Nothing has been said about their developmental fate while they remained in the adult fly. Larval organ anlagen are unable to develop in the abdomen of adult male hosts in the absence of larval ring glands (Bodenstein, 1943). Nevertheless we find in the experiments reported on page 114 that young eye discs left behind in the anterior part of 1st instar larvae develop quite extensively when transplanted into adult male hosts, although no larval ring glands were present. By their growth the discs are usually forced out through the cut surface of the small transplant so that they are finally placed almost or entirely outside of the implant itself. In Figure 4 are shown the two developed eye discs of a transplant which had remained for 14 days in the adult male host environment.

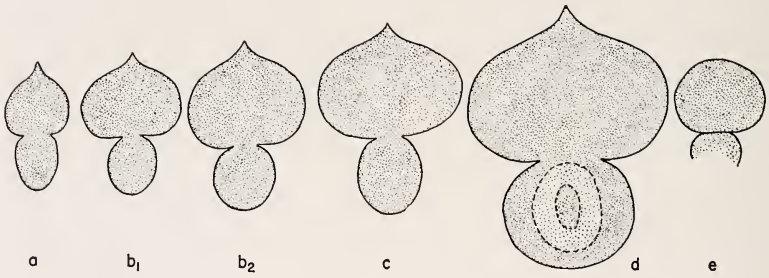

FIGURE 12. a. Eye-antenna disc of a 2nd instar larva shortly before the second molt. b_1 and b_2. Eye-antenna disc which had been transplanted into an adult male host at stage a. Note the increase in growth. c. Eye-antenna disc of very young 3rd instar larva. d. Eye-antenna disc of mature larva. e. Eye disc which had been transplanted at 1st instar stage into an adult male host. (For further explanation, see text.)

Since we know that eye discs of 3rd instar larvae are unable to develop in adult male hosts and find, as described above, that eye discs of 1st instar larvae are able to develop in the same environment, there must be one definite stage in development beyond which the discs are unable to develop. In order to find this critical stage one further experiment was designed and is semi-diagrammatically represented in Figure 12. The size relationships in this figure are based on camera lucida drawings of the same magnification. Figure 12 a shows an eye disc of a 2nd instar larva shortly before the second molt. One may observe that at this stage the distal portion of the disc, the eye disc proper and the posterior portion, the antenna disc, are separated only by a small constriction. Although the eye and antenna discs are bound up together during the whole of the larval life, they become more distinctly separated from one another as development proceeds. A few hours after the second molt the eye and antenna discs have become two definite structures, as illustrated in Figure 12 c. At this stage there is no differentiation recognizable in the antenna discs in total mounts. The eye disc of a mature larva

is shown in Figure 12 d. The considerable increase in size of the eye during the third larval instar is evident if one compares Figure 12 c with Figure 12 d. Moreover, histological differentiation has taken place during this time in both discs. In the mature eye discs the cells have arranged themselves into a definite pattern, which is however scarcely perceptible in total mounts, whereas the differentiation processes in the antenna are visible as oval concentric folds within the disc.

If eye discs of young 3rd instar larvae (Fig. 12 c) are transplanted into the abdomen of adult male flies it is noticed that these discs are unable to grow further. Yet eye discs of old 2nd instar larvae (Fig. 12 a) transplanted in the same manner reach the stage of growth shown in Figure 12 b_1 and b_2 but apparently do not develop further. In comparing the discs in Figures 12 b_2 and c, it may be noticed that both discs are almost the same size. From the foregoing consideration this should be expected, for since the discs of stage "c" are unable to grow in an adult male environment, the younger discs should cease growing in this environment when they reach stage "c." Thus the critical period for the cessation of growth of the eye discs lies between stages "a" and "c," i.e. at about the time the larvae undergo their second molt. Therefore the growth of eye discs transplanted at stages younger than "a" (see experiments above) should never surpass the growth stage "c." This expectation has been fulfilled, for one finds (Fig. 12 e) that the final growth stage of the largest eye disc shown in Figure 4 lies somewhere between the stages of growth represented in Figures 12 a and b.

Discussion

The results described in this paper emphasize the important part played by the ring gland in the larval molting of Drosophila. The molting of larval parts *in vivo* in the abdominal cavity of adult flies is possible only when larval ring glands are added. The speed with which the molts occur is, however, much slower than in normal development, where the two larval molts are accomplished during 4 days. In only one case has the transplant molted twice during the 7 days it remained in the adult host, while the majority of the cases molted only once, although some of them had remained as long as 15 days in the adult host. The slow developmental progress of larval structures in an adult environment was also observed in earlier experiments, and is apparently due to the different metabolic situation within the adult environment (Bodenstein, 1943). In this connection it is of interest to note that the transplant, which had molted twice, was a virilis head part transplanted together with two ring glands into a melanogaster female. It has been established (Bodenstein, 1943) that the melanogaster female environment is much better suited for the growth of organ discs than either melanogaster male or virilis female or male environments. It seems that the same applies to the molting of larval structures. This observation supports a suggestion (Bodenstein, 1943) that the ring gland does not act directly, but rather indirectly in affecting the metabolic level of the host, which then in turn is responsible for the various developmental achievements of the transplanted structures. This hypothesis also assumes that the larval or pupal level is much higher than the highest level induced by ring glands in the adult host (see Bodenstein, 1943, p. 56). If this is correct, it should follow that larval head parts of the first instar should always molt twice in a larval or pupal environment, and that the successive molts should proceed in more rapid succession

in a larval than in an adult environment. The fast rate of normal development, as compared with the very slow developmental rate of the transplanted head parts in adult hosts, is indicative in supporting the "level" conception. The experiments in which larval head segments were transplanted into the abdomen of 3rd instar larvae offer another case in point; for three out of eight cases had already molted twice in 5 to 6 days, while five out of five cases had also molted twice in 7 days. Moreover, in these cases the transplants were under the influence of but one ring gland, that of the host larva, while in the adult hosts two ring glands were present. A similar situation is also found when one compares the second instar larval head transplants in larval and adult host environments. The fact that several transplants in adult hosts had not molted at all, although they had been in their hosts for a considerable length of time, may also be explained by the very low effective level of this environment.

Molting is a process involving many different structures. In normal development there is apparently a marked synchrony between the structural systems involved, in that all of these tissues are ready to perform their molting task at one set time. The same situation, it would seem, prevails in the different organ anlagen systems, as far as their reactions towards pupation are concerned. Yet different organ anlagen vary in their competence to respond to the metamorphosis hormone. These differences, however, are not apparent in normal development, but become noticeable in a modified hormonal environment (see Bodenstein, 1943, p. 55). As far as the molting of head segments in adult flies is concerned, we find the spiracles apparently reacting more readily to the ring gland hormone than the mandibles. For although the spiracles are more difficult to detect, we have cases where it is certain that the mandibles have not molted, while the spiracles have (see Table II). Thus the different systems involved in the process of molting possess different degrees of reacting competence (see Bodenstein, 1943, pp. 53–55).

The number of molts in Drosophila seems to be fixed at two, for 2nd instar head parts transplanted into 3rd instar larvae molt only once, while 1st instar head parts may molt twice although they have remained no longer in the host than the 2nd instar head parts. This seems to be quite unusual in the light of our knowledge of hormone-induced molting in insects (see review Bodenstein, 1942). It might, however, be that the mouth parts used here as a criterion for molting react with greater difficulty than some other structures.

The competence of larval discs

It has been stated previously that in the development of organ discs, the processes of growth as well as of differentiation are controlled by the same hormone. Whether growth or differentiation takes place, depends upon a definite relationship between hormone level and organ competence (Bodenstein, 1943). The observation that young organ discs are able to grow in adult male hosts, but only to a certain stage of growth, still further clarifies the hormone control of Drosophila development. It shows that young discs are very responsive, at least as far as the growth response is concerned, since older discs fail to grow in the same environment. Furthermore these young eye discs grow only to a stage reached by normal eye discs at the time the larva enters the 3rd instar. This fact is decisive, since it shows that the male environment is effective in inducing growth only and does so

solely in very responsive young discs. The question now arises, have these young discs only the competence for a growth response, or are they also able to differentiate under the influence of a very effective hormone level? The answer is provided in an experiment (Bodenstein, 1939a) where young eye discs were transplanted into the abdomen of mature larvae, and thus subjected to a very highly effective level, with the result that they did not reach imaginal completion. Since older eye discs transplanted in the same manner complete their imaginal differentiation in synchrony with the host, it follows that the young discs respond with growth, until they have reached the size above which they can respond with differentiation. By this time, however, the host organs are already so far advanced in their differentiation that the young discs are unable to catch up. In the light of these considerations we might ask what takes place in the eye discs at the time they reach their maximal growth in the adult male fly? The young discs must have acquired the competence to react with differentiation if the appropriate stimulus is present. They are now bi-potent; they react to a low effective level with growth, but might be induced to differentiate, provided the effective hormone level is high enough. That this is the case can be demonstrated experimentally, for an eye disc of a 3rd instar larva will grow considerably before differentiation begins in an adult male environment if supported by two larval ring glands (Bodenstein, 1943). The same eye disc transplanted into a mature larva will, however, cease to grow and begin to differentiate immediately, with the result that finally a small imaginal eye is formed (Bodenstein, 1939a; 1941). This experiment shows clearly that the response of the same organ disc varies according to the hormonal situation. Thus we may conclude that as the eye discs of 3rd instar larvae become older their competence to respond with growth gradually decreases, while at the same time the competence of the differentiation response increases. At the end of larval life the discs are able to respond only with differentiation.

It is of interest to note that our earlier results (Bodenstein, 1943) and those discussed here are confirmed by recent experiments of Vogt (1943). This author, working with *Drosophila hydei,* approached the problem in a different way experimentally. She used as host the rear part of a 3rd instar larva, after the anterior larval part had been removed by means of a ligature. Into these larval rear parts she then transplanted the eye discs of 3rd instar larvae and simultaneously the eye discs plus the nervous system of very young 2nd instar larvae. She found that the older eye discs remained larval (whether growth occurred is not stated) while the young eye discs developed to a stage equivalent to that of an old 2nd instar larva. When, however, she transplanted six ring glands of the young 2nd instar donors simultaneously, the result was different. The young eye discs had become larger, while the old eye discs had begun differentiation. From this Vogt concludes that growth and differentiation are apparently controlled by the same hormone.

Here attention should be drawn to the fact that this critical stage which occurs at about the time of the second molt corresponds closely to critical periods observed in two other sets of experiments. 1. Beadle *et al.* (1938) and Bodenstein (1939b; 1941) observed that Drosophila larvae (melanogaster and hydei) when starved completely before they are 50 and 100 to 120 hours old respectively, i.e. about the time of the second molt, are unable to pupate. 2. Bodenstein (1939a;

1941) transplanted melanogaster eye discs from donor larvae younger than 50 hours into mature larvae and found that these discs were unable to differentiate completely, while older eye discs transplanted in the same manner differentiate to imaginal completion. From all this it becomes evident that at this time a change occurs in the organ discs of the larvae, the nature of which we have discussed above. Whether all the imaginal discs reach this critical stage at the same time is questionable, since one must remember that the different discs, as well as different regions of the same discs (Bodenstein, 1943), respond differently to the same effective level. Whether all discs have such a critical stage is at present unknown, but seems highly probable.

Summary

1. Larval head parts of 1st and 2nd instar larvae without nervous system and ring glands were transplanted into the abdomen of adult male flies, together with two larval ring glands from mature larvae. The transplanted heads molt once or twice, as indicated by the presence of double or triple mouth parts.

2. Head parts transplanted in the same manner, but without larval ring glands fail to molt. This shows that the larval ring gland hormone induces larval molting.

3. Head parts without their nervous system and ring glands were also transplanted into the abdomen of 3rd instar larvae. The transplantations were performed either homo- or heteroplastically. In either case molting was induced. The molting hormone of the ring gland is thus not species-specific.

4. Very young organ anlagen were transplanted together with anterior larval head segments after the removal of the nervous system into adult male hosts. It was found that these organs were able to develop in their new environment in the absence of ring glands. Older organ discs transplanted in the same manner develop however only in the presence of ring glands. These experiments are discussed in relation to hormone level and tissue competence.

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THE DEVELOPMENT OF FUCUS EGGS IN CONCENTRATION GRADIENTS: A NEW METHOD FOR ESTABLISHING STEEP GRADIENTS ACROSS LIVING CELLS[1]

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Introduction

Fucus eggs have been reared in gradients of hydrogen ion concentration (Whitaker, 1938; 1940) and in temperature gradients (Lowrance, 1937), by methods already described. The developmental polarity of the embryo was determined in these cases, and further analysis by means of gradients of known substances and conditions is desirable. Pease (1941) has reared Echinoderm eggs in concentration gradients of enzyme poisons and other substances by placing them on a membrane of scintered glass that separates two solutions. He has been able to associate the determination of the dorso-ventral axis with parts of the cyanide-sensitive respiratory mechanism.

The present paper describes a simple new method for establishing very steep gradients across living cells. It is applicable to a wide variety of types of living cells, and the steep gradients may be either chemical or physical, including temperature and gas pressures as well as various water solutions. The method also permits a relatively exact estimate of the actual concentration or condition at all parts of the cell surface. In the experiments now to be reported, gradients of dinitrophenol were applied to Fucus eggs in August and September, 1941.

Method

The method consists essentially in dispersing living cells in a liquid medium that will gelate when the temperature decreases, and in dipping a ring-like collar of glass into the liquid so that a very thin film is formed in the collar, similar in general to a soap bubble film or to the film formed in a bacteriological loop. The film, which contains living cells, gelates on cooling. The collar is then fitted into a hole in a glass or lucite partition that separates two flowing solutions of different composition, and the thin film thus becomes a part of the partition. The concentration gradient in the film may be very steep because the film is very thin. Solutions at different temperatures, or gas mixtures, may be substituted for the solutions of different composition.

The procedure as actually carried out will now be described in more detail. A drop of sea water containing Fucus eggs[2] was mixed with a drop of 2 to 3 per cent agar-sea water in a small watch glass or other small glass vessel. At the time of the mixing the agar was fluid, but it was close to congealing temperature, having

[1] This work was supported in part by funds granted by the Rockefeller Foundation.

[2] Five to six hours fertilized, in these experiments.

cooled nearly to 37° C. The watch glass was warmed slightly before the eggs and agar were added so that the mixture would not congeal instantly.

Collar-like cylindrical cross sections of an ordinary 2 cm. glass tube had been cut earlier with a glass saw. Each of these glass collars or cylinders was about one-eighth inch long. The actual mixing of the eggs and agar, as described in the preceding paragraph, was done with a glass collar held in the fingers. The mixing must be done very rapidly, as the high temperature is damaging if maintained. The collar was pulled away quickly after the mixing, and this caused a very thin film of agar to form across and within the collar. It was not difficult to remove the collar within 1 to 2 seconds after mixing the eggs with the warm agar, and the thin film cooled almost instantly.

Many films were removed from the collars and thin sections of the films, cut with razor blades, were examined under the microscope. The main central area of a film was found to be quite uniform in thickness. Near the edge the film became suddenly thinner and then flared out into a thick attachment meniscus. The eggs were found to be fairly evenly distributed throughout the agar film, in which they are firmly embedded so that they do not roll or move. The thickness of the film could be varied, with a little practice, by varying the concentration of agar, the temperature of the mixture, and the speed of withdrawal of the collar. It was also found possible by successive dippings to build up a multiple laminated film. The thickness of measured single dipped films varied from 30 to 150 μ, and the range could readily have been extended in either direction. The Fucus eggs average about 80 μ in diameter, and in very thin films they bulged out of the film on both sides. They remained firmly embedded however, and it is therefore presumed that a very thin film of agar covered the bulging surfaces of the eggs. In the experiments to be reported below, it was arranged for the films to be as thick as or slightly thicker than the diameter of the eggs. The films ranged from 80 to 130 μ thick.

Gelatin and gelatin-agar mixtures were also used for films. Gelatin has the advantage of a lower congealing temperature but the gelatin available to us (bacteriological) tended to be somewhat toxic to the Fucus eggs, and the experiments were therefore carried out in agar. Other gels might well turn out to be quite satisfactory.

Once the eggs were embedded in the film in the collar, to subject them to steep gradients it was necessary only to place the film as a sort of window in a partition separating two different solutions. This was done by drilling a 2 cm. hole in a glass plate and grinding one of the collars to fit snugly into the hole. The collar was mounted into the plate before dipping the film and it was to some extent cemented in place with agar-sea water jelly which filled any imperfections in the ground fit. The upper half of a Coplin jar was cut off and discarded. The glass plate bearing the collar (containing the film) was inserted into the lower half of the Coplin jar thereby dividing it into two chambers. The plate fitted in between the fins or corrugations originally designed to hold slides in the Coplin jar. The plate had been cut to fit the slightly tapered jar, and one or more wide rubber bands were snapped around the edge of the glass plate before it was inserted in the Coplin jar. These rubber bands acted as an elastic gasket that sealed the fit when the plate was pushed firmly down into the jar.

It was necessary to maintain equal pressure on the two sides of the film to prevent its rupture. The lower half of the Coplin jar was filled with sea water before the plate was carefully pushed into place. Sea water was then caused to flow into the chamber on one side of the plate, while the experimental solution (e.g. dinitrophenol in sea water) flowed into the chamber on the other side of the plate. The rates of flow were adjusted by means of stopcocks to be about the same on both sides, to minimize pressure difference, and each chamber was drained by means of a levelling siphon leading in both cases into the same small dumping reservoir. The dumping reservoir was a small vial held to the side of the Coplin jar with a rubber band so that its height could readily be adjusted. It drained by overflowing.

Several duplicate apparatuses were set up at the same time, and the two solutions were siphoned into the chambers from large reservoirs. The rate of flow into the chambers was judged to be more than ample to maintain the concentrations in the chambers and at the surfaces of the agar film in spite of diffusion through the film. Concentration at any distance into the film can be estimated on the basis of calculated diffusion.

Fucus eggs embedded in an agar film develop normally when the film is maintained in a moist atmosphere instead of in sea water. The agar gel serves as an adequate medium provided it is prevented from drying out. It is thus possible to subject living cells to concentration gradients of gasses in the agar film by flowing moist gas mixtures through chambers on either side of the film.

In certain later experiments lucite plates were used instead of glass. The lucite was non-toxic and was easier to cut and drill than glass. Rectangular glass vessels were sawed in half across the middle of the long axis, and a lucite plate with flat rubber gaskets was placed between the two halves of the vessel, which were pressed firmly against the plate so that there was no leakage. Solutions flowed into the two halves of the vessel, now serving as the two chambers on either side of the plate.

All experiments were set up in dim red light, after which the Fucus eggs were reared in the dark until the end of the experiments since the point on the surface of the egg at which the rhizoid forms is affected by the shorter wave-lengths of the visible spectrum. The eggs were kept at 15° C., except during the embedding process.

Results

After the embedded Fucus eggs had been fertilized 24 to 30 hours, the glass plates were removed from the Coplin jars and placed flat in small dishes containing sea water. The film was inspected microscopically to make counts of rhizoids pointing up or down, i.e. toward or away from the surface of the film facing the lens of the microscope. After a count had been made the plate and film were turned over and a count was made from the other side as a check. Counts made from the two sides were averaged. They usually agreed quite well. In some cases thin strips of the agar film bearing eggs were cut with a razor, and these strips were then examined on edge under the microscope to observe the direction of rhizoid formation and growth.

In the present experiments the agar films were 80–130 μ thick. The pH of the media ranged in different experiments within the limits: 8.2 ± 0.05. A stock solution of dinitrophenol (10^{-3} M) in sea water was adjusted to the pH of the prevail-

ing sample of sea water before being added into the final sea water-dinitrophenol solution. The pH of sea water and dinitrophenol-sea water did not differ measurably in an experiment.

The results are shown in Table I. The controls, with sea water on both sides of the film, serve to indicate the accuracy of counting since the control counts should approximate 50 per cent toward one side. It is clear that a high proportion of the

TABLE I

The percentages of rhizoids formed on the sides of eggs subjected to the highest concentration of dinitrophenol are shown in the right hand column. The controls indicate the accuracy of the method of counting, since approximately 50 per cent of the rhizoids in the controls would be expected to form toward one side of the membrane

Experiment number	Concentration dinitrophenol M.	Number of eggs	Rhizoids toward dinitrophenol %
1	5×10^{-5}	119	95
2	4×10^{-5}	124	99
3	4×10^{-5}	58	98
4	3×10^{-5}	123	99
5	3×10^{-5}	79	87
6	3×10^{-5}	108	73
7	3×10^{-5}	175	95
8	3×10^{-5}	92	74
9	3×10^{-5}	132	95
10	3×10^{-5}	101	79
			Rhizoids toward one side, %
Control	0	104	53
Control	0	55	51
Control	0	70	58

rhizoids formed on the sides of the eggs subjected in the gradients to the higher concentrations of dinitrophenol.

Experiments have not yet been carried out to show precisely the effects of dinitrophenol on the respiration of Fucus eggs, but on general grounds it might be expected to affect respiratory rate.

SUMMARY AND CONCLUSIONS

1. A new method is described for establishing across living cells very steep concentration gradients of chemical substances in solution, or for establishing temperature gradients. The cells are embedded in a thin jelly membrane that separates two different flowing solutions.

2. When fertilized Fucus eggs, embedded in a thin agar membrane, are reared with sea water on one side and $3\text{–}5 \times 10^{-5}$ M dinitrophenol in sea water on the other side of the membrane, a high proportion form rhizoids on the sides toward the higher concentration of dinitrophenol.

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PARAMECIUM BURSARIA: LIFE HISTORY. I. IMMATURITY, MATURITY AND AGE[1]

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Introduction

The discovery of mating types or sex types in the ciliate infusoria has made it possible to breed and cross these Protozoa as readily as higher organisms. This has made possible under favorable conditions a renewed study of the problems of youth, age and death, particularly in relation to conjugation. These problems form the subject of the investigation of which a first installment is presented here.

The unit for examination in such studies is the clone rather than the single cellular individual. We deal with youth, age and death of clones, not of single cells only. The clone consists of all individuals derived by vegetative fission from a single ex-conjugant. The age of the clone is properly reckoned from the time of separation of the two ex-conjugants of the ancestral pair.

A number of investigators, beginning with Maupas (1888, 1889) have reported that there is, following the separation of the conjugants, a period of immaturity, during which multiplication by fission occurs, but conjugation does not occur. This is followed, according to these reports, by a period of maturity during which conjugation may occur. The period of maturity is said to be followed by a period of decline or degeneration, often spoken of as age or senescence. According to Maupas (1888) conjugation during this period of decline results in the death of the conjugants. The decline itself, without conjugations, also ultimately results in death. G. N. Calkins was long the outstanding representative of this general conception of the life history of the ciliate, though he did not, I believe, report that conjugation during the period of decline results in death.

However, a number of investigators have shown that in some of the ciliate infusoria and in certain other Protozoa, a clone may, under favorable conditions, continue indefinitely to multiply by fission without decline, degeneration or other indications of senescence (Woodruff, Metalnikoff, Bělař, Hartmann, Beers, Dawson and others). A detailed review of these investigations, with references to the literature, is found in Jennings, 1929.

[1] This work was aided by grants from the Carnegie Corporation and from the Committee on Research in Problems of Sex, of the National Research Council.

However, after these demonstrations as before, it continues to be observed that clones of ciliates, cultivated for long periods in the laboratory undergo gradual decline and ultimate degeneration and extinction. The graph of vitality, as indicated by the rate of fission, shows a gradually descending course, as illustrated for many cases in Jennings, 1929. The experience of the present writer in recent long-continued culture of *Paramecium bursaria* has been of this type.

What is the nature of this gradual decline and degeneration, in the cases where it occurs? What is its relation to environmental conditions, to ageing, to conjugation, to other developmental or genetic processes? It is with these and related questions that the present investigation deals. A method of detecting and following the progressive decline of clones was discovered in the fact that the mortality after conjugation commonly increases with the age of the conjugating clones. This has made possible a detailed study of the problems.

The fact that clones of *Paramecium bursaria* show, beginning immediately after separation of the conjugants, successive periods of immaturity, adolescence, maturity, and decrepitude in age, has been mentioned in previous papers (Jennings, 1939; 1939a; 1941; 1941a; 1941b; 1942). The phenomena have now been subjected to extensive cultural and experimental analysis. In presenting the results, the different periods of life will be successively taken up. Clones now or recently in the laboratory have been cultured for many different periods, up to eight years. Some of these clones are derived from individuals collected in nature; others were produced by conjugation in the laboratory. These numerous clones furnish abundant favorable material for examination of the pertinent questions.

The investigation has been devoted mainly to the genetic or intrinsic factors involved, though later installments will deal to some extent with the action of environmental factors. The method of work has been largely that of inducing conjugations within or between known clones, particularly at different ages, and following the history of the clones produced at these conjugations. Other results of such matings, particularly as to the inheritance of sex types or mating types, have been presented in detail in two previous papers (Jennings, 1941; 1942); those results form a foundation for much of the work to be presented in the series here begun.

The conditions in *Paramecium bursaria* that are particularly favorable for this type of work may be briefly recapitulated. Any variety of the species is differentiated into several mating types or sex types. One variety has eight sex types; two others have four each; a fourth variety seemingly has but two. In any clone all the individuals are of the same sex type, save in the infrequent cases of differentiation of a clone into two sex types (see Jennings, 1941). Mature clones of a given sex type conjugate readily with any of the other sex types of that variety (when their individuals are mingled together) but not with clones of their own sex type, nor with clones of other varieties. The readiness to conjugate and the mortality after conjugation vary with the laboratory age and with certain other conditions, as will be set forth.

The investigation has been an extensive and long-continued one. The author has been able to devote his time and energy to it exclusively, so that it has been the hope and design to devote so much time and attention to each problem that arose as to clear it up fully. This hope has been realized, I believe, for certain aspects of

the work, but also many questions have been raised that will require further work. The investigation deals with all the various periods of the life history of clones, but the chief problem for study has been age and natural death. The present first installment deals mainly, however, with the periods of immaturity and maturity, and with a general survey of the phenomena of ageing and death. Later installments will present detailed experimental investigations of ageing and death and of their relations to conjugation.

Culture Methods

The organisms were cultivated in the lettuce infusion impregnated with *Flavobacterium brunneum* (see Jennings, 1939; 1942). In the earlier years the alga *Stichococcus bacillaris* was added to the infusion (Jennings, 1939). In later years the alga was not used, as the organisms flourish equally well without it. Also for certain later years the infusion employed was diluted to one half the concentration described in 1939, but in the long run this does not work as well as the more concentrated infusion. For months the organisms flourish as well as they do in the concentrated infusion, but decline begins earlier in the dilute infusion. Therefore from 1943 the more concentrated infusion was used, as in the earlier years.

If the infusorians are under culture in a small vessel, such as a Syracuse dish, or in the deep depressions of thick culture slides, they continue to multiply vigorously if transferred to new culture fluid about once a week. If the cultures are left unchanged for considerably longer periods, some of them continue to flourish, but in most of them the animals cease to multiply after a time, and they become thin, at the same time decreasing in number. Why some old cultures continue to flourish, while most do not, is obscure. The infusoria may live in a depressed condition in the old cultures for long periods, reviving and becoming vigorous, with resumption of multiplication, when transferred to fresh infusion. There are indications that this environmentally-induced depressed condition is due in some considerable measure to the unfavorably altered condition of the infusion, possibly to deleterious products of metabolism from either themselves or the bacteria on which they feed, rather than to mere lack of food. In any case it is important to distinguish such temporary and purely environmental depressions from the depressed conditions of the clone that is largely independent of present environment, as described later. Whether however there is a connection between the two—whether long-continued extrinsic environmental depression may tend in time to induce intrinsic or genetic depression—may remain for the present an open question.

Clones differ greatly in their resistance to unfavorable cultural conditions. Some die out quickly under conditions in which others continue to exist and from which they recover when the conditions improve. Particular clones, indeed, differ at different periods of their lives in their resistance to unfavorable conditions: a matter which forms in large measure the subject of the present investigation.

The clones that have been cultivated for long periods in our laboratory have as a rule been subjected at intervals, particularly in the earlier years, to periods of environmental depression. This appears to be of importance in connection with the long periods of immaturity shown by some of the clones, as will be set forth in a later section.

Special methods will be described in connection with the phenomena in relation to which they are employed.

Immaturity: Its Dependence on Cultural Conditions

For a period of time after conjugation the descendants of the ex-conjugants neither form clots nor conjugate, even though brought under favorable conditions into intimate contact with individuals of different sex type, belonging to the same variety. This period of sexual immaturity varies greatly in length in different clones, even among those produced by conjugation of the same two parental clones. Immature periods varying in length from a minimum of twelve days up to some years have been observed (see Jennings, 1939, pp. 214–215). There is among our clones one ("403*a*1") that was produced October 20, 1937 (by self fertilization of a clone of Variety I); it still does not conjugate (July 1943) though more than five years old. This clone that has never become mature has a well developed micronucleus (T. T. Chen, personal communication); it has now (1943) passed into a period of depression.

The great variation in length of the period of immaturity, taken in connection with the fact that many or most of the clone cultures were subjected at times to the depressing conditions mentioned above, suggested that possibly the age at which sexual maturity is reached depends to some extent on the cultural conditions. This was tested in the following way:

By conjugation between two clones a large number of pairs was obtained. From these pairs were derived many ex-conjugant clones (two from each pair). Each of these ex-conjugant clones was divided, after it had multiplied to some extent, into two parts. One part was kept in vigorous multiplication by changing and renewing frequently the culture fluid, and by keeping the number of individuals small; this is known as the rapid portion (R). The other part of each clone was subjected to unfavorable conditions, which interrupted or made very slow the multiplication by fission; this is known as the slow portion (S). This was done by allowing the cultures to become crowded, and the culture medium to become old. There were thus genetically identical sets of R and S clones, with many clones in each set. After a period of this diverse culture, the two sets of clones were restored to the same favorable cultural conditions. Here they were allowed to multiply, and at intervals the clones of both sets were tested in the usual way to determine if they were sexually mature. That is, the two divisions (R and S) of each clone were tested each with the four mature sex types of the variety (Variety I). Clones that were mature reacted by forming clots and pairs with three of the four sex types; those that were immature did not form clots or pairs.

Two extensive experimental cultures of this type were carried through; both showed that the cultural conditions in which a clone has lived do indeed affect the age at which it becomes mature. Each experiment lasts for many months and requires several or many successive tests of each clone, at intervals of weeks or months.

The first experiment was begun May 9, 1941, at which time 24 pairs were obtained from the conjugation of the two clones 39 and 44, respectively of the sex types D and B of Variety I. (Clone 39 was collected December 23, 1939 at Monterey, California; clone 44 March 6, 1941, at Westwood Village, Los Angeles,

California.) After separation the two ex-conjugants of each pair were isolated, so that there were 48 ex-conjugant clones. Each was allowed to multiply separately in fresh culture medium until May 20 when a considerable number of individuals was present in each of 46 of the clones (two of the 48 ex-conjugant clones were non-viable).

Now each of the 46 ex-conjugant clones was separated into two parts: a "rapid" part, designated R and a "slow" part, S. There were now therefore 92 separate cultures, two for each of the 46 clones. Each "rapid" culture, R, was begun with but ten individuals, while its corresponding "slow" culture, S, included at the beginning a large number of individuals (the remainder of the clone). The rapid cultures were kept in vigorous multiplication; every second day four individuals of each were employed to carry on the culture, and these were transferred to abundant fresh culture medium, the rest of the culture being discarded. In the slow cultures of the 46 clones, on the other hand all the individuals were left in each case on the depression slide and were there allowed to multiply till they became so numerous that reproduction nearly or quite ceased and the individuals became thin. As the culture fluid evaporated, fresh weak fluid was added, at intervals of several days, but there was in these slow cultures no transfer to fresh slides or infusion. Thus the slow cultures were kept for a long period in a depressed condition. Their individuals remained normal in appearance, but were slender and did not become numerous.

At later periods both the rapid and the slow were placed in fresh fluid in Syracuse dishes, and were allowed to multiply there till they became sufficiently numerous to be tested for maturity. At the time of testing, therefore, the two sets were living under the same conditions and were both multiplying well. Does the earlier different history of the two make a difference in the age at which maturity is reached?

The first test was made June 6 to June 23, 1941, when the clones were about one month old. The first 12 clones were tested from each of the two sets of cultures, the tests being carried out in the usual way. None were yet mature; no clots or pairs were produced.

The next test was August 7 to 16, 1941. Five of the slow cultures had succumbed and died out; the corresponding rapid cultures of the same clones were discarded. This left 41 clones, each represented by a rapid and a slow culture. All those in the rapid cultures except four had now become sexually mature, as shown by their reaction (production of clots and pairs) with the test clones. But none of the slow cultures reacted; none was mature.

Thus the difference in cultural conditions had produced a great difference in the age at which the organisms became sexually mature. At the age of 3 months those parts of the 41 clones that had been kept vigorously multiplying were all mature except four, while their genetic duplicates that had been subjected to depressing conditions were all still sexually immature.

The four clones of the rapid set that did not react in the August tests became mature in October. At the age of 5 months all the 41 clones in their rapid cultures were mature.

From the time of the August tests the slow cultures were tested at intervals of about one month. Some additional cultures died out, so that 33 clones were tested

in the slow cultures in October; none were yet mature. In the latter half of November 1941, the slow cultures of 30 clones were tested, one had now become mature. No additional culture of the slow set had become mature at tests in December 1941, January 1942, February 1942, or March 1942.

In July 1942, 14 months after conjugation, the still surviving cultures of the slow set were again tested. Only eight clones were now represented in the eight living cultures of the slow set. Of these, four were now fully mature, while two others were becoming mature, since they reacted with two of the four sex types of the variety. The other two cultures were still immature.

Thus while clones kept in vigorous multiplication all become mature at the age of 3 to 5 months, the same clones subjected to depressing conditions became mature only at 10 to 14 months or later.

It appeared desirable to repeat this experiment. The second experiment began October 22, 1941, at which time were isolated 54 pairs, produced by the conjugation of two clones derived from two different pairs of the "rapid" set of the experiment just described. On October 27 each of the 108 ex-conjugant clones from these 54 pairs was divided into two parts, a "rapid" lot (R) and a "slow" lot (S). The rapid set was cultured, as in the previous experiment, in such a way as to keep the individuals multiplying rapidly. The slow set (duplicate of the rapid) was subjected, as before, to crowding and starvation. There were thus 108 clones in each set.

The cultures of the "slow" set were subjected to depressing conditions, in this case, for but 18 days, till November 14, 1941. On this date all the surviving clones, R and S, were placed in Syracuse dishes, and thenceforth all were cultivated in the same way, in the usual dilute lettuce infusion. The point to be determined is whether the cultivation of one of the duplicate sets under depressing conditions for 18 days, while the other set was vigorously reproducing, has made a difference in the age at which sexual maturity comes on.

In this second generation experiment the mortality of the clones was much greater than in the previous generation. Of the total 216 ex-conjugant cultures (sum of the R and the S), only 91 survived to be tested. These included the same clone in both sets in 37 cases (37 R and 37 S that were genetically identical). In addition there were 5 clones that were represented in the R set but not in the S set, while 12 other clones were represented in the S set, but not in the R set. That is, 42 clones were represented in the R set, 49 in the S set, and 37 of those in each set were the same clones.

In the 37 clones that were common to the two sets, the rapid culture became mature in every case before the slow culture of the same clone. In 22 of the 37 cases only the rapid culture became mature within the 8 months during which the experiment was continued, the same clones in the slow cultures remaining immature to the end. In the other 15 clones of the 37 that were represented in both sets of cultures, both cultures became mature, the rapid culture in every case a month or more earlier than the same clone in the slow set.

Up to the end of February 1942, when the clones were a little more than 4 months old, 42 clones of the R set had been tested, and 36 were found to be mature; that is, in the rapid cultures 85.7 per cent of the clones were mature at the end of 4 months. At the same date 46 of the 49 clones in the slow set had been tested, of

which 14 were found to be mature, so that but 30.4 per cent of the clones in the slow cultures were mature at the age of 4 months. At the end of June, when the clones were 8 months old, 40 clones were mature of the 42 in the rapid cultures (95.2 per cent) while at the same time but 20 of the 49 clones in the slow cultures were mature (40.8 per cent).

Thus these two long-continued experiments give the same result. Clone cultures that are kept vigorously multiplying, without starvation or crowding, or staleness of the culture medium, become mature much earlier than the same clones in cultures that have been subjected for a considerable period to the depressing effects of the conditions just named. Those kept vigorously multiplying usually become mature in 3 to 4 months, though there is much variation among them, and in some the immature period is much shorter. Those that have been subjected to depressing influences such as starvation, crowding and staleness do not become mature till they are 10 to 14 months of age, or older. In both the experiments described above a number of the clones thus subjected to depressing conditions did not become mature during the time the experiments continued.

In these phenomena we see temporary differential action of diverse environments producing in the stocks differences that continue for months of vegetative reproduction after the differential environmental action has ceased. In the second experiment described above the effectively diverse environmental conditions lasted for but 18 days, but the two sets thus diversely acted on retained the induced diversity for at least 7 months. The induced diversity is transmitted in vegetative reproduction for many successive generations.

It has been suggested verbally to me that possibly the time of becoming mature depends on the number of vegetative generations that have passed since conjugation. Depressing conditions decrease the number of vegetative generations passed through in a given period of time, and would therefore, if the above suggestion is correct, lengthen the time to maturity. But in view of the great difference in the time of becoming mature induced by a relatively short period of depressing conditions, the above suggestion appears not probable, and it is more likely that the action of depressing conditions is more direct, producing physiological changes that delay the attainment of maturity more or less independently of the number of vegetative generations that have passed. The matter is one that is worthy of precise and detailed experimental study, as indeed is the entire phenomenon of the relation of the attainment of maturity to environmental conditions.

The facts shown by the experiments are of practical importance for the investigator. For many purposes it is necessary to cultivate the ex-conjugant clones until they are mature, in order that needed crosses may be made. The time required for this is greatly shortened if the clones are kept continuously in vigorous multiplication. It appears probable that the frequent very long periods of immaturity in my own work mentioned in earlier papers, were due to the fact that in their culture the clones were at times subjected to depressing conditions.

There is an important additional relation observed on comparing the rapid and slow subdivisions of the clones. Though clones produced by different pairs are frequently of different sex types, the two subdivisions of any single ex-conjugant clone are always finally of the same sex type, in spite of the fact that they have been cultured differently and have become mature at widely different periods. In the

two experimental cultures of which account is given above, there were 21 ex-conjugant clones in which the sex-type was determined for both the rapid or early maturing, and the slow or late maturing subdivisions. Of the 21 ex-conjugant clones 13 were of sex type A, five were B and three were C. In all cases both subdivisions of any clone were of the same sex type. This agrees with all other evidence in showing that the sex type is determined genetically and is not ordinarily altered by changed environmental conditions.

Transitional Period from Immaturity to Maturity

In the life of most clones there is a period of transition, during which the ex-conjugant clone reacts sexually only in sporadic individuals, and in some cases with only one or two of the sex-types of the variety to which it belongs. This transitional period may last for weeks. Details, with many examples of the sporadic sex reactions, have been given in the third report on these investigations (1942, pages 196–199). Rarely the transitional period is short, or possibly entirely lacking, the clone suddenly acquiring the typical strong reaction in all (or most) of its members, with the usual clot formation and resultant numerous pairs.

Maturity

The sexually mature period is characterized in typical cases by the fact that when clones of different sex type are mingled, there occurs the strongly marked clotting followed by formation of numerous pairs (Jennings, 1939).

It is notable, however, that the strength of the tendency to clot varies greatly in different clones. Some clones when mixed form at once large clots, like those photographed in the 1939 paper. Others form but small clots, containing only a few individuals (three or four or less). In some clones only a few individuals take part in the clotting, while in others all are active in the sexual reaction. Some clones do not react at once when mixed, but do react later. Some do not react on the day the mixture is made, but react (strongly or weakly) the next day.

These differences in the tendency to clot resemble those described by Moewus in certain flagellates, which Moewus has correlated with different genetic constitutions. (For summaries of this work of Moewus, the paper of Sonneborn, 1941, may be consulted.) These phenomena are worthy of detailed study in the ciliates.

The period of sexual maturity lasts for several years, and is followed by a period of decline which forms the chief subject of the present investigation.

A remarkable phenomenon is to be observed at times in mature clones. When two clones of different sex type (but of the same variety) are mingled, strongly marked clotting usually occurs, the individuals coming into intimate contact; but in some cases no conjugated pairs are finally produced. The clotting occurs during the day; toward evening the clots break up, not into pairs, as in the normal case, but into separate single individuals. In the normal case, after clotting has occurred, many united pairs are found to be present in the mixture the next morning, before the new clotting of the second day has begun. These pairs remain in union for 36 hours or more. But in exceptional cases no pairs are present the next morning. On the second day clotting may occur again, but as before no lasting pairs are

formed. Thus the first stages of the mating reaction occur, but the final stages do not; conjugation is not completed.

A similar phenomenon has been described by Sonneborn (1942) in certain clones of *Paramecium aurelia*. In *Paramecium bursaria* such clotting without formation of pairs may at times characterize many clones of a collection. Such cases are the following: In February 1943 collections were made from ponds in the Botanical Garden of the University of California at Los Angeles; also from ponds in the Municipal Park at Beverly Hills. A considerable number of these clones showed clotting without formation of lasting pairs, presenting opportunity for a study of the phenomena.

From the two collections 46 clones were isolated and cultivated. These included representatives of all the four sex-types of Variety I. Three clones were of sex type A, nine of type B, 24 of type C, and ten of type D. Representatives of each clone were tested with all clones of the three types to which the clone under test did not belong. In all cases clots were formed, but a considerable number of the mixtures did not form lasting pairs. All clones of sex types A and D (13 clones in all) formed clots and pairs in the normal way, when mixed with clones that were capable of forming pairs. Of the nine clones of the B type, four were normal, forming clots and pairs in the usual way, while five ordinarily did not form pairs, even when mixed with clones that were themselves thoroughly normal. All these form clots as usual, though no pairs. Of the 24 clones of C type, seven formed clots and pairs in the usual way, while 17 formed clots but did not form pairs. Thus of the 46 clones 24 formed clots succeeded by pairs in the usual way, while 22 clones formed clots but did not form pairs (save in isolated instances; see next paragraph).

A peculiar feature of the phenomena is that certain of the clones that as a rule do not form pairs may in certain instances form one or two pairs. This occurred in mixtures in which in normal reactions there would be found as many as a hundred or more pairs. Such isolated pairs were observed in five of the clones that commonly formed no pairs. Three of them were of type C, while two were of type B. In each of four of these five clones but a single lasting pair was observed, though there were for each clone several mixtures in each of which many pairs would in the normal case be formed. In the fifth clone ("BH130") no pairs were formed in mixtures with any of the 37 clones of different sex types that belong to these collections; but three pairs were formed when individuals of this clone were mixed with certain "tester" clones (of types A and D), that had been selected because of their strong sexual reactions.

To determine whether a micronucleus is present in the clones, particularly in those that do not form pairs, a cytological examination was made by Dr. T. T. Chen. This examination covered 41 of the 46 clones in the collection mentioned above; it included 19 of the clones that do not ordinarily form pairs, and 22 of those that do. A micronucleus was found to be present in all the clones that regularly form pairs; also in 15 of the 19 clones that clot but do not form pairs. Four clones were without micronuclei, and all of these belonged to the group that clot but do not ordinarily form pairs. Two of them had produced an isolated pair or two in certain mixtures.

Absence of a micronucleus thus usually, though not always, prevents pair forma-

tion, though it does not prevent clotting. But other conditions may prevent formation of pairs, since 15 clones that had micronuclei did not form pairs.

The absence of a micronucleus does not prevent the manifestation of the sex type. Of the four clones without micronuclei, three belonged clearly to sex type B, while the fourth was a well defined type C.

In the other 37 clones the micronuclei varied considerably in form, size and stainability. But no special characteristics appeared peculiar to the 15 clones that did not form lasting pairs.

The cytological changes in this clotting without conjugation, or "formation of temporary pairs" have been examined by Dr. T. T. Chen, who will report on them in a separate paper.

Age, Mortality, and the Consequences of Conjugation

We have in the laboratory many clones of the different varieties of *Paramecium bursaria*. In January 1943 there was under culture a reserve stock of 264 clones, in addition to many recent ex-conjugant clones that were under immediate study. The clones are of many different ages. Some have been under cultivation for about eight years (April 1943). Others vary from six years to but a few months or days of laboratory cultivation. Many of the clones were collected in nature, from diverse parts of the United States or abroad. Others were produced in the laboratory by the conjugation of pre-existing clones.

Many of these clones that at first flourished vigorously have since declined in vigor, with alteration of many of their life phenomena. Some have died out, in spite of the greatest care in cultivation. Many of the old clones at conjugation produce pairs of which the majority die. These observations formed the starting point of the present investigation.

Symptoms of Degeneration as Clones Become Old

Our oldest clones, Nos. 1 (or "m") and 2 (or "l") were collected April 18, 1935, at Alexandria, Virginia. At the time they came into my hands, in June 1937, they had been maintained for two years in the laboratory by Dr. T. T. Chen. What their age may have been at the time of collection there is of course no way of knowing, so that the total age of each is unknown. The two have shown in the later years depression or degeneration manifested in a number of different ways. Their history is instructive.

When they came into my hands in June 1937 Clones 1 and 2 were vigorous in vegetative reproduction, but showed a high mortality at the time of conjugation. The two were of different sex type (No. 1 of type C, No. 2 of type A), and were bred together in June and July 1937. From their mating 142 pairs were obtained, yielding, after separation of the conjugants, 284 ex-conjugants. Of these, descendants of but 18 ex-conjugants survived and formed clone cultures; that is, but 6.3 per cent of the ex-conjugant clones survived.

The clone No. 1 was divided into many cultures which were cultivated separately. In January 1938 certain of these cultures yielded pairs by self differentiation and self fertilization. There were 118 of these pairs from the selfing of clone Number 1, yielding 236 ex-conjugants. Again the mortality was excessively high,

only six of the 236 surviving and forming ex-conjugant clones. Thus but 2.5 per cent of the ex-conjugants from the selfed clone No. 1 survived.

Not all of the cultures No. 1 underwent self differentiation and self fertilization. Those that did so contained in consequence individuals of the two mating types C and B, which conjugated, yielding pairs as just set forth. But several cultures remained of the pure original type C. In order to insure that the cultures should contain only this type, new cultures were started from single individuals, of the mating type C.

In February 1940 it became apparent that the cultures of clone No. 1 were less flourishing than those of other clones. By October of that year all cultures of this clone had become scanty, and when new cultures were seeded with a number of individuals of the clone there was little multiplication. The contrast with other clones in this respect was striking. Individuals of abnormal form made their appearance in the cultures of clone No. 1. A number of the cultures died out.

From October 17 to October 28, 1940, a period of 12 days, comparative isolation cultures were carried on of this clone No. 1 and of three other clones (see Table I). These were for the purpose of comparing the rate of fission and the

TABLE I

Paramecium bursaria; rates of multiplication and frequency of deaths in certain clones, in comparison with Clone 1, for the period October 17 to October 28, 1940.

Each clone consists of 24 parallel lines cultivated for 12 days. The number of fissions in the 24 different lines during the 12 days is summarized; also the total number of deaths of lines in each clone of 24 lines.

Clone 1 (Laboratory age 66 months). Number of fissions in the different lines varies from 0 to 7. Number of deaths, 14.

Clone 2 (Laboratory age 66 months). Number of fissions, 17 to 23. Number of deaths, 4.

Clone 6 (Age 40 months). Number of fissions, 20 to 27. Number of deaths, 2.

Clone 36 (Laboratory age 11 months). Number of fissions, 27 to 30. Number of deaths, 1.

frequency of deaths in clone 1 with those of the three other clones. Twenty-four lines of each clone were cultured on depression slides. Each of the 24 lines of each of the four clones was begun as a single individual in one of the depressions of the slides. Each was allowed to multiply for 24 hours; then the number present was recorded and a single individual from each depression was transferred to a new slide and fresh fluid, and allowed to multiply for 24 hours as before. This was repeated for each of the 96 lines throughout 12 days. At the end of the 12-day period there were records for the 24 lines of each of the four clones. In Table I are given the number of fissions in the lowest and highest line of each clone; in other words, the range of variation in fissions for the 24 lines of the clone. Table I also gives the number of deaths in the 24 lines of each clone during the 12-day period. This number of deaths was obtained as follows: After the daily transfer of a single individual to a new slide, sometimes this individual died, ending the line. The line was then continued by substituting an individual from one of the other lines of that clone. The "number of deaths" in Table I shows how many times this occurred in the 24 lines of each clone.

The clones compared with clone 1 in Table I are the following:

Clone 2 was collected at the same time and place as clone 1, and hence was of

the same laboratory age. It was not vegetatively depressed at the time of the cultures of Table I.

Clone 6 was derived from a pair resulting from the conjugation of clone 1 with clone 2, June 18, 1937. It is therefore younger than its parent clones 1 and 2 by somewhat more than 2 years.

Clone 36 was collected at Los Angeles, November 18, 1939.

Thus some of the lines of clone 1 did not divide at all during 12 days, and the most vigorous line divided but seven times. In contrast, the other three clones, living under exactly the same conditions, multiplied at the rate of one, two, or more fissions daily, in each line. The clone 1 shows many deaths and hardly multiplies at all.

During November 1940 it became increasingly difficult to keep the cultures of clone 1 alive. There were many such cultures, some on slides, some in Syracuse dishes. Some were cultivated in the dilute lettuce infusion, some in the more concentrated, some with algae, some without. Under all these conditions other clones multiplied vigorously. But one after another the cultures of clone 1 died out, until on January 26, 1941 the last culture died and the clone 1 became extinct.

A similar downward course has been followed by certain other clones. Some have become completely extinct; others still exist as weak scanty cultures that are kept alive only with difficulty. All this has occurred under conditions in which other clones flourish. Some examples may be mentioned.

The clone "McD3" was collected near Baltimore, Maryland, February 7, 1938. It was long one of the most vigorous of the clones in the laboratory, and was employed as the chief tester of sex type M, Variety II. But it became weak and degenerate, and for many months in 1942 and 1943 it was kept alive only with difficulty. It finally died out, in all its cultures, in March 1943.

A considerable number of clones of Variety III were obtained, some from North Carolina, February 25 and March 20, 1938; some from Provincetown, Massachusetts, July 28, 1938. By crosses among the clones from North Carolina many additional clones were obtained. Some 24 clones were kept under culture as a reserve stock. Now after more than 5 years almost all of these stock clones have died out. Before final death they were for many months in bad condition, multiplying little and showing hardly any tendency to sexual reaction. The few clones that remain are in bad condition and will doubtless soon die. As Variety III is not known to occur on the Pacific slope and has rarely been collected elsewhere, the loss is a serious one, leaving the laboratory without testers for Variety III.

A peculiar variation in these histories is shown by the clone known as S of Variety II. It was collected in the spring of 1937. For years it flourished and was employed as a tester of the sex type J. In 1940 to 1943 it became very weak and many of its cultures died out. All of those which were kept rapidly multiplying died, but a certain old quiescent culture, in which the animals were thin and multiplied little or not at all, has remained alive. Attempts to bring its members to rapid multiplication by additions of fresh culture fluid results in their immediate death.

Many other cases of decline or degeneration of clones after they had long been under culture have been followed in this laboratory. A large number of stock cultures were long kept on hand. These were transferred weekly to new cultures.

For a long time they flourished, now more than half of them have died out (June 1943).

Age of Clones in Relation to the Readiness to Conjugate and to Fertility and Mortality in Conjugation

Observations on decline in old clones such as set forth above left in many cases the further impression that old clones may conjugate but that the mortality after such conjugations is greater than usual, many of the ex-conjugants dying without the production of long lived clones. Maupas in his great papers of 1888 and 1889 was left with a similar impression. He gives many instances of high mortality at conjugation in Stylonychia, in Onychodromus, in Leucophrys, in Didinium, in Spirostomum. In some of these cases the stocks were known to be old; in others this was uncertain. In some cases Maupas attributed the high mortality to the supposed or known fact that the conjugating animals were close relatives. In view of these impressions and the accounts given by Maupas, an extensive investigation was undertaken of the relation both of age and of inbreeding to mortality at conjugation. The results of this work will be presented in full in later papers.

Since the time of Maupas the only work bearing on this precise matter appears to be that on mortality at endomixis in *Paramecium aurelia*, carried out mainly by Sonneborn or under his inspiration, and published by Jennings and Sonneborn, 1936, by Gelber, 1938, and Pierson, 1938. At the time the work was done it was not known that "endomixis" is a form of conjugation, or closely related to it, as "autogamy." But Diller (1936) showed for *Paramecium aurelia* that in "endomixis" two nuclei unite, both produced of course by the single individual, so that the process is a close form of self-fertilization; and this was confirmed genetically by Sonneborn (1939). (Whether in all cases endomixis includes such autogamy is extensively discussed by Woodruff, 1941.) Jennings and Sonneborn (1936) showed that lines which omit endomixis die, and that many die at the occurrence of endomixis; also that "It appears that the longer endomixis is omitted the greater the proportion of individuals that die when they undergo it" (p. 419). This relation to the time elapsed since the foregoing endomixis was examined statistically by Gelber (1938) and Pierson (1938). They showed that the older the lines, reckoned from the last endomictic period, the greater the proportion of deaths at endomixis. Comparison of their results with those on conjugation in *Paramecium bursaria* will be presented in later installments of the present investigation.

Readiness to conjugate in old clones: In clone No. 1, above described, it was found that the individuals were ready to conjugate long after the time when the clone had become weak; it continued till very shortly before the death of the clone. In mixtures of clone No. 1 (sex type C) with clones of types A, B and D, clotting occurred January 5, 1940; though all cultures of clone No. 1 died out January 26 of that year. It was notable, however, that though typical clotting occurred, in but few cases were pairs formed and conjugation completed. From the abundant clotting of January 5 but four firmly united pairs persisted till the next morning. Three of them separated that morning, indicating that conjugation had not been consummated. The fourth separated at the normal time, later, but of the two ex-conjugants one died without fission, the other divided but once, then both its descendants died.

Other old clones formed clots and later pairs, when mixed with clones of other sex types. The fate of these pairs is to be taken up later.

Thus old clones of Variety I undergo clotting and pairing with other sex types up to a short time before their death from age; a result that agrees with the observations of Maupas on other species.

Age in relation to mortality at conjugation: In most epidemics of conjugation there is a certain amount of mortality among the ex-conjugants or their immediate descendants. When the clones that conjugate are old the percentage of mortality is high, as seen in certain instances cited above.

The relation of mortality at conjugation to age of the conjugating clones presents opportunity for experimental study of the nature and progress of ageing. It has therefore been subjected to extensive and intensive investigation. The data on this and the conclusions to be drawn are to be presented in papers soon to appear.

Summary

The life history of clones of *Paramecium bursaria* shows successive periods: (1) a period of sexual immaturity, during which sexual reactions and conjugation do not occur: (2) a transitional period during which weak sexual reactions occur in a few individuals: (3) a period of maturity, in which sexual reactions are strongly marked and the individuals conjugate readily: (4) a period of decline, ending in many (or all?) cases in death.

The length of the period of immaturity and the time of attainment of maturity depend on the cultural conditions. If the animals are kept rapidly multiplying, under the best of nutritive conditions, maturity comes on early; if they are subjected to periods of starvation or other depressing conditions, maturity comes on much later or not at all.

Ex-conjugant clones that are kept vigorously multiplying become mature in most cases at the age of three to five months, though cases have been observed of much earlier maturity, the earliest observed age of maturity being 12 days.

Ex-conjugant clones subjected for some time to depressing conditions become mature (even after restoration to favorable conditions) only at the age of 10 to 14 months. Certain clones have lived for years without becoming mature.

If single ex-conjugant clones are divided into two cultures, one subjected to conditions favorable to rapid multiplication, the other to unfavorable conditions, the two parts show these same differences. The part kept under favorable conditions matures months before the other part.

Subjection to depressing conditions for but short periods (18 days) delays maturity for months.

Thus temporary differential action of diverse environments produces in clones differences which persist through months of vegetative reproduction.

In the period of maturity, clotting and conjugation *en masse* are commonly produced when cultures of clones of different sex type are mixed. But in certain clones clotting occurs without the completion of conjugation; dense clots occur, but no pairs are formed.

The period of maturity lasts for several years. It is followed by a period of decline. In this period fission becomes slower; abnormalities appear; many individuals die, so that the cultures become scanty and finally die out completely.

Clones have been cultivated in the laboratory five to eight years, finally showing degeneration and death.

During the period of decline conjugation may occur up to near the very end. But conjugation of aged stocks results in the death of most or all of the ex-conjugants.

The relation of age to mortality at conjugation presents many features of interest, and gives opportunity for study of the nature and progress of ageing. This matter is to be presented in later contributions.

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THE EFFECT OF FOOD CONTENT AND TEMPERATURE ON RESPIRATION IN PELOMYXA CAROLINENSIS WILSON [1]

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Introduction

Pelomyxa carolinensis Wilson (*Chaos chaos* Schaeffer) is an organism favorable for the study of cellular functions. It is one of the largest of the amoeboid forms, it can be cultured easily in the laboratory, and it is relatively simple in structure and physiological activity. Although it has been available from stock culture since 1937, apparently no studies have been made on the respiration of this organism; in fact, very few investigations have been made on respiration in any of the amoeboid forms.

Emerson (1929), using Barcroft-Warburg manometers, found that the oxygen consumption of *Amoeba proteus* at 20° C. amounts to 0.16 mm.^{3} per mm.^{3} of cell substance per hour. Howland and Bernstein (1931), using a modification of the capillary tube method devised by Kalmus (1928), found that the oxygen consumption of *Actinosphaerium eichhornii* at 20° C. is of the order of 1,100 mm.^{3} per hour per million organisms. They did not express the rate of oxygen consumption per unit volume of protoplasm.

The following investigations were carried out to ascertain the rate of respiration in *Pelomyxa carolinensis* under various conditions of nutrition and at different temperatures.

Material and Methods

The organisms used in these experiments were from a strain which has been cultured in the laboratory for several years in the manner described by Belda (1942). The pelomyxae were grown in Hahnert's (1932) solution, but prior to each experiment the organisms were kept for about a week in a culture medium buffered to maintain a hydrogen-ion concentration of pH 6.8. To keep this value constant, a high concentration of phosphates was required. Pelomyxae, put into experimental solutions containing only potassium and magnesium compounds, quickly disintegrated. However, the addition of calcium chloride served to counteract the toxic effects of the potassium ions, and the pelomyxae grew well in this medium. The formula which was finally adopted is given in Table I.

The pelomyxae were kept in glass finger bowls containing 150 ml. of buffered solution. Food was supplied for the organisms by adding portions of a centrifuged culture of *Paramecium caudatum* until there were about 600 to 700 paramecia per ml. Under these conditions the pelomyxae ingest from one to three paramecia

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TABLE I

Buffered culture solution for Pelomyxa carolinensis

K_2HPO_4	80 mg.
KH_2PO_4	80 mg.
$CaCl_2$	104 mg.
$Mg_3(PO_4)_2 \cdot 4H_2O$	2 mg.
H_2O (redistilled)	1,000 ml.

within an hour, and continue feeding as long as any paramecia remain. Specimens of Pelomyxa were removed from the finger bowls at intervals for respiration studies.

The rate of oxygen consumption and carbon dioxide elimination was measured by means of a Barcroft-Warburg apparatus. The shaking rack held seven manometers and flasks, of which one was used as a thermo-barometer. Preliminary tests had shown that there was no measurable difference in the rate of oxygen consumption between pelomyxae tested in flasks which contained 100, 200, or 300 organisms. In the experiments as carried out, groups of either 100 or 200 specimens were used in each test.

To measure the rate of oxygen consumption, 0.4 ml. of 10 per cent KOH was put into the inset and 0.3 ml. of 3N HCl into the onset of each flask. To measure both the rate of oxygen consumption and of carbon dioxide elimination, the manometers were paired and 0.4 ml. of distilled water was put into the insets of half of the flasks and 0.4 ml. of 10 per cent KOH into those of the other half. The water bath of the apparatus was kept at the temperature selected for each experiment with a variation of not more than $\pm$ 0.05° C.

The manometers were mounted on a shaking mechanism which was operated at the rate of 124 complete cycles per minute through an amplitude of 3 cm. This amount of motion provided a sufficiently rapid exchange between the gases and liquids in the flasks.

After the manometers and flasks had been put into place with the stopcocks open, the shaking mechanism was run for one hour. At the highest and lowest temperatures used in the experiments, this period was extended to 2 hours. By this time the temperature in the flasks was equal to that of the water bath and practically all of the carbon dioxide originally present in the flasks had been absorbed. The stopcocks were then closed, and the level of the liquid in each manometer was recorded at intervals of one hour.

The volume of the pelomyxae was calculated by measuring specimens in a volumescope. This apparatus, devised by Chalkley (1929) and modified by Belda (1942), consists essentially of a capillary pyrex glass tube into which a pelomyxa can be put. The bore of the tube is such that the pelomyxa assumes a cylindrical shape with rounded ends. The length of the specimen can be measured accurately by means of a compound microscope provided with a camera lucida. The volume of the specimen can be calculated by using the equation

$$V = \pi r^2 l + 4/3\pi r^3$$

in which r is the radius, both of the cylindrical part of the pelomyxa and of each of the rounded ends, and l is the length of the cylindrical part.

The surface area of a pelomyxa varies considerably, depending on the number of pseudopodia which may be extended. The average surface area is, however, approximately equal to that of a specimen which has the shape of an elongated cylinder (Belda, 1943). Accordingly, the equation

$$A = 2\pi rl + 4\pi r^2$$

in which the symbols have the same value as in the preceding equation, is appropriate for calculating the approximate surface area.

In conducting the experiments, 20 specimens of average size were selected from those to be used in each test. These specimens were taken up with a capillary pipette and measured in the volumescope. At the conclusion of each test 20 specimens were again selected and measured. From these measurements the average volume and surface area was calculated. There was no appreciable variation between the average values obtained at the beginning and at the end of each experiment.

Results

I. Effect of food content on respiration

In much of the work previously reported on cellular respiration in the Protozoa, the food content of the organisms was not considered. However, Lund (1918) observed that there was a decrease in the rate of oxygen consumption in paramecia which had been starved for 20 hours, and Leichsenring (1925) observed a decrease of 23 per cent in the rate of oxygen consumption in paramecia which had been starved for 24 hours.

In order to ascertain what types of pelomyxae would be suitable for studying the effect of different temperatures on the rate of respiration, preliminary experiments were carried out on specimens in varying states of nutrition. The first tests were made with what are designated well-fed specimens. About 1000 pelomyxae together with numerous paramecia were put into finger-bowls containing buffer solution as described under Material and Methods. After 24 hours each pelomyxa had several large food vacuoles containing partly digested paramecia. Several hundred pelomyxae of average size were removed with a capillary pipette and washed in three separate portions of sterile buffer solution. Five milliliters of sterile buffer solution containing either 100 or 200 pelomyxae were put into each manometer flask; and 5 milliliters buffer solution without pelomyxae were put into the flask of the thermo-barometer. In the first three tests both oxygen consumption and carbon dioxide elimination were measured. The results are presented in Table II.

In the first three tests, the rate of oxygen consumption was 15,530 mm.^{3}, and of carbon dioxide elimination 13,510 mm.^{3}, per hour per million organisms. Calculated from these values, the respiratory quotient is 0.87.

For the second part of the experiment, pelomyxae were put into finger bowls containing buffer solution without paramecia and left for one week without food. These are designated starved pelomyxae. At the end of this time all food vacuoles have disappeared, and the average volume of the specimens is about 25 per cent less than that of well-fed pelomyxae. The average rate of oxygen consumption of

starved pelomyxae per million organisms was found to be about 65 per cent less than that of well-fed pelomyxae; however, the rate of oxygen consumption per mm.^{3} of cell substance showed a decrease of only 54 per cent. In the first three tests with starved pelomyxae, the average rate of carbon dioxide elimination per million organisms was 3,490 mm.^{3} per hour, a decrease of 73 per cent from that of well-fed specimens. The respiratory quotient was 0.56.

In the third part of the experiment, pelomyxae were put into finger bowls containing buffer solution, and large numbers of paramecia were added as in the first part of the experiments. After 3 days the pelomyxae had ingested nearly all the paramecia, but still contained numerous large food vacuoles. The pelomyxae were

TABLE II

Rate of oxygen consumption in starved, "normal," and well-fed specimens of Pelomyxa carolinensis
Temperature: 25° C. Hydrogen-ion concentration: pH 6.8

Type of organism	Test number	Number of organisms in each test	Duration of experiment	Average volume of one million organisms in mm.^{3}	Average rate of O_2 consumption in mm.^{3} per hour per million organisms	Average rate of O_2 consumption in mm.^{3} per hour per mm.^{3} of cell substance
Starved	1 to 3	100	5 hours	27,600	6,200	0.188±0.052
	4 to 6	200	6 hours		4,890	
	7 to 9	200	6 hours		4,340	
					Mean: 5,210±1,450	
"Normal"	1 to 3	100	5 hours	35,580	9,730	0.275±0.032
	4 to 6	200	7 hours		9,950	
	7 to 9	200	12 hours		9,730	
					Mean: 9,800±1,140	
Well-fed	1 to 3	200	5 hours	36,800	15,530	0.407±0.040
	4 to 6	200	7 hours		15,270	
	7 to 9	100	7 hours		14,120	
					Mean: 14,970±1,500	

left in the finger bowls for 3 more days, but no more paramecia were added. At the end of this time most of the organisms still had two or three small food vacuoles. These organisms are designated "normal" pelomyxae. The results of all these experiments are combined in Table II. This table shows that the rate of respiration in *Pelomyxa carolinensis* is closely correlated with the amount of food material present in the cytoplasm, for there is a progressive increase in the rate of oxygen consumption in starved, "normal," and well-fed specimens.

II. Effect of temperature on respiration

In the previous experiment, there was less variation in the rate of oxygen consumption of "normal" pelomyxae than of starved or well-fed specimens. Accordingly the remaining tests were all made with "normal" specimens.

A number of tests were made to measure the rate of oxygen consumption of

Pelomyxa at temperatures between 15° C. and 40° C., using 5° increments. Groups of either 100 or 200 organisms in 5 ml. buffer solution, treated as described above for "normal" pelomyxae, were tested. The results are given in Table III. This table shows that the rate of oxygen consumption for Peolmyxa increases in nearly

TABLE III

Rate of oxygen consumption in Pelomyxa carolinensis *at different temperatures. Hydrogen-ion concentration, pH 6.8*

Each figure under oxygen consumption per million organisms represents the average for three tests except those taken at 25° C. which are averages for four tests.

Temperature	Test number	Number of organisms used	Duration of experiment	Average volume of one million organisms in $mm.^3$	Average approximate surface area/ million organisms in $mm.^2$	Average rate of O_2 consumption in $mm.^3$ per hour per million organisms	Average rate of O_2 consumption in $mm.^3$ per hour per $mm.^3$ cell substance
15° C.	1 to 3	200	7 hours	34,950	583,000	4,660	0.144±0.024
	4 to 6	200	7 hours			5,080	
	7 to 9	100	4 hours			5,280	
	10 to 12	200	5 hours			5,130	
						Mean = 5,040± 850	
20° C.	1 to 3	200	7 hours	36,800	607,900	7,070	0.191±0.022
	4 to 6	200	5 hours			6,920	
	7 to 9	100	5 hours			7,150	
						Mean = 7,050± 827	
25° C.	1 to 4	100	12 hours	36,800	607,900	10,250	0.244±0.028
	5 to 8	200	10 hours			8,750	
	9 to 12	200	6 hours			8,170	
	13 to 16	100	12 hours			8,540	
	17 to 20	200	10 hours			9,930	
	21 to 24	200	6 hours			8,380	
						Mean = 9,010± 910	
30° C.	1 to 3	200	12 hours	35,580	590,000	10,990	0.372±0.049
	4 to 6	100	12 hours			14,455	
	7 to 9	200	5 hours			14,420	
						Mean = 13,244±1,760	
35° C.	1 to 3	150	5 hours	34,950	583,000	19,010	0.507±0.044
	4 to 6	200	6 hours			17,530	
	7 to 9	100	6 hours			16,630	
						Mean = 17,749±1,540	

linear ratio from 15° to 25° C. Between 25° and 35° C. the rate of oxygen consumption increases more rapidly, but again in nearly linear ratio.

The lowest temperature at which tests were carried out was 15° C. At this temperature the pelomyxae showed a tendency to become spherical. The amount of protoplasmic streaming and the rate of locomotion were considerably diminished.

At the higher temperatures, the pelomyxae were more active. At 35° C. the rate of locomotion was rapid; long, narrow pseudopodia were extended in many directions, so that the organisms were nearly stellate in shape. All the specimens remained alive during these tests.

In nine of the 12 tests which were made at 40° C. the pelomyxae died during the first hour. In the remaining three tests, the organisms survived long enough for a single reading of the manometers to be made. The average rate of oxygen consumption for the three tests, each made with 100 pelomyxae at 40° C., was 28,300 mm.^{3} per hour per million organisms. This rate is much higher than that at 35° C. Because of the high mortality of the organisms at 40° C., the results obtained are probably not comparable to those at lower temperatures, and they are not included in the table.

The temperature coefficient (Q_{10}) for the rate of oxygen consumption of Pelomyxa between 15° and 25° C. is 1.7; between 25° and 35° C. the Q_{10} is 2.1.

Discussion

The rate of oxygen consumption in well-fed pelomyxae is 2.16 times that of starved specimens. This difference is similar to that found by Lund (1918) in comparable types of Paramecium. He found that specimens tested after having been fed boiled yeast suspension used up from two to three times as much oxygen as specimens tested after having been starved.

The rate of oxygen consumption of pelomyxae kept for 7 days without food is 32 per cent less than that of "normal" specimens kept for 3 days with practically no food. Similarly, the rate of oxygen consumption of pelomyxae kept for 3 days with practically no food is 32 per cent less than that of well-fed pelomyxae. This difference is comparable to a decrease of 29 per cent in paramecia after having been kept for 72 hours without food, as measured by Leichsenring (1925). The close agreement between the results for Paramecium and Pelomyxa may, however, not be significant, since the two organisms differ greatly in their structure and activity.

The published data for respiration in Paramecium are based on the quantity of oxygen consumed and carbon dioxide given off per organism. They do not take into account the volume nor the surface area of the specimens. At least approximate values for the volume and surface area of Paramecium can be calculated by means of the equations given by Fortner (1925). These, however, require measurements of the length and diameter of the specimens.

That the volume of the organisms ought to be considered, at least in tests made on organisms differing in food content, can be seen from Table II. The decrease in the rate of oxygen consumption per unit of protoplasm for starved pelomyxae is relatively less than the decrease per organism.

Apparently the only measurement of the respiratory quotient of an amoebid organism previously reported is that of Emerson (1929), who found a value of slightly less than 1.0 for *Amoeba proteus*. However, the R.Q. for other free-living Protozoa has been measured. Emerson (1929) found a value of slightly less than 1.0 for *Blepharisma undulans*. The R.Q. for Paramecium, as found by various investigators, is as follows: Amberson (1928), 0.69; Root (1930), 0.62; and Mast, Pace, and Mast (1936), 0.72. These values are all similar to that of

Pelomyxa carolinensis. The significance of measurements of the R.Q. for various Protozoa has been discussed at length by Jahn (1941).

The rate of oxygen consumption per hour per mm.^{3} of protoplasm for Pelomyxa at 20° C., namely 0.191 mm.^{3}, is slightly higher than the rate of 0.16 mm.^{3} for *Amoeba proteus* found by Emerson (1929). Considering possible errors in the measurements of the volume of the organisms, this difference may not be significant. The rate of oxygen absorption per unit of surface area in *Amoeba proteus* is, however, much less than that in Pelomyxa. An exact comparison is not possible because Emerson did not measure the surface area of Amoeba. But since the volume of Amoeba is only about 1/50 that of Pelomyxa (Belda, 1942), the amount of surface area of Amoeba per mm.^{3} of protoplasm is many times greater than that of Pelomyxa.

Howland and Bernstein (1931) did not calculate the rate of oxygen consumption per unit volume of protoplasm in *Actinosphaerium eichhornii.* However, an approximate value can be obtained. The average diameter of *Actinosphaerium eichhornii* is between 200 and 300 micra. Since the organisms are approximately spherical in shape, the volume can readily be calculated from the diameter. The average rate of oxygen consumption at 20° C. is 0.26 mm.^{3} per mm.^{3} of protoplasm for specimens of Actinosphaerium having a diameter of 200 micra, and 0.11 mm.^{3} for specimens having a diameter of 300 micra. These values are of the same order of magnitude as those for Pelomyxa at 20° C.

The rate of oxygen consumption of Pelomyxa at 15° C. is 25 per cent less than that at 20° C. This difference is nearly equal to that observed in Paramecium by Leichsenring (1925). She measured the rate of respiration in paramecia at 20° C., then lowered the temperature to 15° C., and found that the rate of respiration had decreased 30 per cent.

Leichsenring also tested paramecia at 20° C., then increased the temperature to 35° C., and found an increase of 35 per cent in the rate of respiration. This is much less than the increase in rate found in the present investigation over the same range of temperature, since the rate of oxygen consumption in Pelomyxa is 135 per cent higher at 35° C. than at 20° C. It should be kept in mind that Leichsenring observed the effects of change in temperature on the same culture of Paramecium, whereas in the present experiment different lots of Pelomyxa were used at the different temperatures. However, Barratt (1905) found that the rate of CO_2 production of Paramecium at 27–30° C. was more than twice that at 15° C. This difference is nearly equal to that difference in the rate of oxygen consumption of Pelomyxa between these temperatures.

The Q_{10} value of 1.7 between 15° and 25° C. for pelomyxa is less than the Q_{10} value of 2.1 found by Lwoff (1933) for Paramecium between 13° and 23° C. On the other hand, the Q_{10} value of 2.1 for Pelomyxa between 25° and 35° C. is higher than the value of 1.5 for Paramecium between 23° and 32° C. found by Kalmus (1928) and also by Lwoff (1933).

Summary

1. The rate of respiration in *Peolmyxa carolinensis* at 25° C. is closely correlated with the amount of food material present in the cytoplasm.

2. The rate of oxygen consumption at 25° C. was found to be 0.244 ± 0.028

mm.^{3} per hour per mm.^{3} cell substance and does not differ greatly from that of *Amoeba proteus* and *Actinosphaerium eichhornii*. The rates were less at low temperatures (15° C.) and greater at high temperatures (35° C.).

3. Temperatures above 35° C. are usually lethal to Pelomyxa, although in several tests the oxygen consumption at 40° C. for one hour was obtained.

4. The respiratory quotient is much lower for starved specimens (R.Q.: 0.56) than for well-fed specimens (R.Q.: 0.87) and is approximately equal to that of other free-living Protozoa.

5. As the temperature is increased, the rate of oxygen consumption increases, but the rate of increase becomes progressively greater.

6. The temperature coefficient for the rate of respiration in *Pelomyxa carolinensis* is nearly the same as that in Paramecium, varying from 1.7 between 15° and 25° C. to 2.1 between 25° and 35° C.

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THE EFFECT OF TEMPERATURE ON THE RATE OF DISAPPEARANCE OF CAUDAL BANDS IN FUNDULUS HETEROCLITUS [1, 2]

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Introduction

It has been known since the work of Wyman (1924) that a transverse cut made near the base of the caudal fin of the killifish, *Fundulus heteroclitus* Linn., severs the nerve supply to the melanophores in the area posterior to the incision. In a white-adapted fish, this operation results in the immediate appearance of a sharply defined black band of completely dispersed melanophores, extending distally from the cut to the posterior edge of the tail.

From the experiments of Mills (1932a) and Parker (1934a) evidence has been accumulated for the hypothesis that the formation of the band is due to the mechanical stimulation, by cutting, of the melanophore-dispersing nerves to this region of the tail. Moreover, the tendency of such a band to be maintained over a period of one or more days is believed to be due to the prolonged though gradually diminishing activity of the momentarily stimulated dispersing fibers (Parker, 1934a).

If a fish with a caudal band is allowed to remain upon a white illuminated background, however, the stripe gradually blanches, the process starting at the edges and progressing inward toward the central region as an increasing number of melanophores concentrate their pigment to assume the appearance of those in normally innervated parts of the tail. This sequence of fading was first reported by Mills (1932b), who regarded it as evidence for the hypothesis that the blanching of caudal bands is due to a concentrating neurohumor secreted by the terminals of concentrating nerves in adjacent regions of the tail and capable of diffusing through the tissues into the denervated area to produce its characteristic effect upon the dispersed melanophores. This hypothesis has been actively supported by several investigators during the past decade, and is now quite generally accepted as the explanation for the blanching of caudal bands in Fundulus and in other teleosts.

The following investigation was undertaken in an attempt to determine the effect of temperature on the rate of disappearance of these caudal bands in Fundulus. A brief study of this type has been made upon the normal paradise fish, *Macropodus opercularis,* by Dalton and Goodrich (1937). These authors found that in each of four animals disappearance of the band required about one half the

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time at 29° C. that it did at 20° C.[4] No other experiments of this sort have been encountered in the literature.

Material and Methods

The experiments to be reported were performed upon *Fundulus heteroclitus*. Its hardiness and availability, together with its conspicuous melanophore responses, made it an especially favorable object for the type of studies involved. Preliminary observations soon made it apparent that the more uniform size and distribution of caudal melanophores in females facilitated the accurate determination of the disappearance of caudal bands, and consequently they were employed exclusively throughout the investigation.

The fish were seined, trapped, or caught with dip nets in the brackish waters in the area of Woods Hole. Healthy females, three to four inches in length, with undamaged caudal fins were then brought to the laboratory where they were used immediately or stored temporarily in a large sink provided with running sea water. This stock of fish, as well as those used in experiments involving a period of several days, was fed every other day on the meat of the clam, *Venus mercenaria* or the edible mussel, *Mytelus edulis*.

Two constant temperature baths were employed in controlling the temperature of the water in which the fish were kept during the experiments. These baths were copper-lined tanks with capacities of approximately 5 cubic feet. Each was filled with tap water which was kept in continuous circulation to insure uniformity of temperature throughout the bath. The temperature of this water was controlled by competition between the cooling effect of a regulated stream of ice water or tap water (depending upon the temperature to be maintained) and the heating effect of a submerged resistance unit controlled by a mercury-toluene thermoregulator through a vacuum tube relay operating on A. C. With proper adjustment of the thermoregulator and the incoming stream of water, it was possible to maintain the temperature of the baths to within extreme limits of $\pm 0.5°$ C. of the desired level over the range employed in these experiments.

A wire shelf suspended 7 inches below the water level in each bath provided support for three flat-bottomed aquaria, 10 inches in diameter and 8 inches in height, which were painted white on the outside and filled with sea water to the level of the water in the bath. Each of these served as a container for four or five fish during the experiments and was aerated by a gentle stream of washed air from a small electric compressor. Illumination was provided by a 60-watt electric light bulb equipped with a white reflector and suspended 15 inches above the surface of the water of each bath. Several trial experiments were performed to determine the advisability of covering each bath with a black tent to eliminate diurnal fluctuations of light intensity. This precaution was found to be unnecessary. Moreover such an arrangement increased the difficulty of maintaining the lower temperatures, and consequently was not employed.

Caudal bands were produced according to the method of Wyman (1924), by making a transverse cut of appropriate length at the base of the tail, approximately one millimeter posterior to the last row of scales. Each animal was allowed to

[4] When calculated from their data, the mean Q_{10} for the group is found to be 2.27.

adapt itself completely to the white background and to the temperature at which the experiment was to be run. The head and trunk were then carefully wrapped in moist cheese cloth and the animal was laid on its right side in a large Petri dish filled with white paraffin. The protruding caudal fin was spread slightly and held flat against the paraffin by the operator's left forefinger while the cut was made. "One-ray bands" were produced by cuts one mm. in length, which severed a single fin ray and approximately half of each adjacent interray. "Two-ray bands" were produced by cuts twice this length, and included two rays, the interray between them, and approximately half of each interray bounding their outer sides. As already noted by many investigators, the large majority of these operations are unaccompanied by any disturbance of blood supply to the denervated area except for the region immediately surrounding the cut.

After the operation each fish was carefully measured from the tip of its snount to the conspicuous arc of blood vessels (Fries, 1931) at the base of the tail. The length to the nearest millimeter was then recorded, together with a sketch indicating the shape and other characteristics of the tail and the position of the band. Such a procedure proved very valuable for insuring future identification of the individual.

After this treatment the fish was returned to its white aquarium in the constant temperature bath, where the band was allowed to fade. The early progress of fading was followed by macroscopic observations at various intervals. Unhealthy animals as well as those whose tails became injured in any way to affect the band were always discarded as soon as they were noticed. When the band became rather faint, subsequent observations were made in so far as possible at intervals of two hours. More frequent observations were generally impractical because of the injurious effects of excessive handling and inability to perceive definite changes in the state of most bands over intervals of less than two hours.

Final stages of blanching were always observed with the aid of a binocular dissecting microscope (10X oculars, 32 or 48 mm. objectives), a method which was found to be more reliable than the use of the unaided eye. Since the blanching is a gradual process, it is of course impossible to determine the exact end point by observations made at intervals of time. Consequently, the mid-point between the time of the last observation in which pigment dispersion was manifested by any of the band melanophores and the first one in which all of these cells had concentrated their pigment as completely as those in the normally innervated regions of the tail was taken as the best estimate of the time of complete fading. Thus, if a band were still very slightly evident 24 hours after its formation, but had completely disappeared by the time of the next observation two hours later, the blanching time would be recorded as 25 hours. Confirmatory observations were then continued for several hours.

Results

Preliminary. Before proceeding with experiments on the relation of temperature to the rate of disappearance of caudal bands, studies were made to determine whether or not an individual Fundulus manifests a more or less characteristic reaction time at a particular temperature level. The advisability of such a study was emphasized by the fact that a great deal of variation had been found to occur among different individuals under the same environmental conditions. Thus, for instance,

in a group of 52 Fundulus with one-ray bands the blanching time at 20° C. varied from 16 to 197 hours; in a group of 17 at 25°, it varied from 13 to 138 hours; and in a third group of 48 at 30°, it varied from 4 to 66 hours.

In determining the degree of constancy of blanching time for an individual, a single one-ray band was produced and its time of disappearance recorded. The fish was then allowed to remain in its white aquarium, and after a few hours a second band was made by cutting another ray a few millimeters above or below the faded area. The time required for disappearance of this second band was then determined without reference to that required by the first one.

It was found that, with few exceptions, the time required for the disappearance of the two bands was remarkably similar. In the majority of cases the difference lay between zero and 2 hours. For a group of 25 fish at 30° C., the mean difference in time of disappearance of the first and second bands was 0.54 ± 1.07 [5] hours. From these data the significance of the mean difference can be estimated from the probability value (P) corresponding to the familiar "t" criterion.[6] For this experiment $t = 0.505$, corresponding to a P value of something over 0.55. Thus, the chances of such a difference being exceeded through errors of random sampling are greater than 55 per cent, and consequently it cannot be regarded as significant. Similar results were obtained with smaller numbers of fish at 15°, 20°, and 25° C. and it was therefore concluded that at a given temperature level there is no significant difference in the blanching time of two one-ray caudal bands produced at different times upon the same fish.

It might be of interest to note that one set of determinations was carried out in such a way as to compare the time of blanching of one-ray bands in sea water and in fresh water at 30° C. During the fading of the first band the fish were kept in sea water, but this was replaced by ordinary tap water for the second run. Of eight individuals successfully carried through the experiment, the mean difference in blanching time of the two bands was 0.44 ± 0.63 hours. The probability of such a difference being exceeded through errors of random sampling was found to be 0.5076 (50.76 per cent) and it is therefore apparent that the rate of disappearance of bands in fresh water is not significantly different from that in sea water.

Temperature. With these preliminary studies in mind, the problem of determining the effect of temperature on the rate of the fading reaction was undertaken. In carrying out these experiments, the time required for blanching at one temperature was determined as usual, and the fish was then transferred to the second bath, the temperature of which was regulated at 10° C. higher or lower than that of the

[5] Standard error of mean. In this paper, standard deviation has been calculated from the formula $s_x = \sqrt{\frac{\Sigma x^2}{N} - \bar{x}^2}$ (Treloar, 1939, p. 55), in which s_x represents the standard deviation; Σx^2 the sum of the squares of each variate; $\bar{x}^2$ the square of the mean; and N the number of individuals in the series. The standard error of the mean has been calculated as $\text{S.E.}_{\bar{x}} = \frac{s_x}{\sqrt{N-1}}$ (Treloar, 1939, p. 138).

[6] Calculated from the equation $t = \frac{\bar{d}}{\text{S.E.}_{\bar{d}}}$ (Treloar, 1942, p. 54), in which $\bar{d}$ represents the mean difference between the pairs of values, and $\text{S.E.}_{\bar{d}}$ the standard error of that mean difference. Corresponding values of P were obtained from Treloar's (1936) extensive table of probability in terms of t.

first. The animal was allowed to adapt itself gradually to the new temperature. After a few hours a new band was produced above or below the site of the original and the time of its disappearance determined. In view of the preliminary work described above, any significant difference in the time required for disappearance of the two bands could be reasonably ascribed to the change in temperature.

Table I shows the results obtained in 17 successful cases in which the first band was allowed to fade at 15° and the second at 25° C. In the column under the

TABLE I

Time in hours required for blanching of one-ray caudal bands at 15° and at 25° C. on the same fish. First run at 15°, second at 25°

Range, interval during which final blanching occurred; Mp, mid-point of that interval; S.D., standard deviation; S.E., standard error of mean.

Fish	Run 1: 15°		Run 2: 25°		Difference Mp_{15}–Mp_{25} (hrs.)	Q_{10}
	Range (hrs.)	Mp_{15} (hrs.)	Range (hrs.)	Mp_{25} (hrs.)		
1	88–90	89	62–66	64	25	1.39
2	74–76	75	42–44	43	32	1.74
3	44–46	45	22–24	23	22	1.96
4	114–116	115	56–58	57	58	2.02
5	44–46	45	20–22	21	24	2.14
6	268–276	272	124–126	125	147	2.18
7	98–100	99	38–48	43	56	2.30
8	98–100	99	38–48	43	56	2.30
9	190–192	191	66–68	67	124	2.85
10	172–182	177	54–56	55	122	3.22
11	170–172	171	52–54	53	118	3.23
12	76–78	77	18–24	21	56	3.67
13	184–186	185	46–48	47	138	3.94
14	88–90	89	20–22	21	68	4.24
15	182–186	184	40–42	41	143	4.49
16	212–216	214	46–48	47	167	4.55
17	146–148	147	26–28	27	120	5.44
Mean	133.76		46.94		*86.82	3.04
S.E.	±15.65 hrs.		±6.08 hrs.		±12.06 hrs.	±0.285
S.D.	62.61 hrs.		24.32 hrs.		48.23 hrs.	1.14

* $t = 7.20$; $P = <.0001$.

heading "Fish" the animals have been arranged arbitrarily in order of increasing values of Q_{10}, recorded in the last column. It can be seen that in every case the blanching reaction required much less time at the higher temperature, the difference being designated in the appropriate column. From 133.76 ± 15.65 hours at 15° the mean time required decreased to 46.94 ± 6.08 hours at 25° C. The value of t calculated from the mean difference is beyond the tabulated range, indicating a probability of less than 1 : 10,000 that such a difference would be exceeded through errors of random sampling.

More informative than the actual difference in time required at the two tem-

perature levels, however, are the Q_{10} values given in the last column of the table. These were found to vary about a mean of 3.04 ± 0.285, indicating that on the average the rate of blanching of caudal bands was approximately trebled by increasing the temperature from 15° to 25° C.

The results obtained upon 14 fish in which the first one-ray band was allowed to fade at 20° and the second at 30° C. are shown in Table II. Here again the

TABLE II

Time in hours required for blanching of one-ray caudal bands at 20° and at 30° C. on the same fish. First run at 20°, second at 30°

Range, interval during which final blanching occurred; Mp, mid-point of that interval; S.D., standard deviation; S.E., standard error of mean.

Fish	Run 1: 20°		Run 2: 30°		Difference $Mp_{20}-Mp_{30}$ (hrs.)	Q_{10}
	Range (hrs.)	Mp_{20} (hrs.)	Range (hrs.)	Mp_{30} (hrs.)		
1	35–36	35.5	22–24	23	12.5	1.54
2	36–44	40	24–26	25	15	1.60
3	39–41	40	21–23	22	18	1.82
4	37–39	38	19–21	20	18	1.90
5	84–86	85	38–48	43	42	1.98
6	36–40	38	18–20	19	19	2.00
7	43–45	44	21–23	22	22	2.00
8	51–55	53	25–27	26	27	2.04
9	22–24	23	10–12	11	12	2.09
10	72–74	73	34–36	35	38	2.09
11	78–80	79	36–38	37	42	2.14
12	46–48	47	20–22	21	26	2.24
13	84–93	88.5	26–28	27	61.5	3.28
14	56–58	57	14–16	15	42	3.80
Mean [7]	52.93		24.71		*28.21	2.18
S.E.	±5.48 hrs.		±2.30 hrs.		±3.93 hrs.	±0.166
S.D.	19.79 hrs.		8.32 hrs.		14.17 hrs.	0.60

[7] Discrepancy of 0.01 between difference of means and mean difference due to rounding off figures at second decimal place.

* $t = 7.18$; $P = <.0001$.

rise of 10° produced a marked increase in the rate of the reaction in every case, the mean Q_{10} value being 2.18 ± 0.166.

A similar relation of temperature to the rate of fading was found in a series of seven Fundulus in which one two-ray band was allowed to disappear at 20° and the second at 30° C. The mean time required at the lower temperature was 126.79 ± 11.31 hours, whereas that at the higher level was 63.29 ± 8.24 hours, the mean difference being 63.50 ± 6.14 hours. It will be noted that the blanching time of these bands at each temperature was much longer than that of the one-ray bands at the same level (cf. Parker, 1934b). The Q_{10} values varied about a mean of 2.09 ± 0.16, however, indicating that in these bands, as in the narrower ones, fad-

ing occurs approximately twice as rapidly when the temperature is raised from 20° to 30° C.

Table III shows the results of another experiment which was also carried out at 20° and at 30° C., but with the temperature sequence reversed. In this case the first determination of blanching time was made at 30° and the fish were then

Table III

Time in hours required for blanching of one-ray caudal bands at 20° and at 30° C. on the same fish. First run at 30°, second at 20°

Range, interval during which final blanching occurred; Mp, mid-point of that interval; S.D., standard deviation; S.E., standard error of mean.

Fish	Run 1: 30°		Run 2: 20°		Difference $Mp_{20}-Mp_{30}$ (hrs.)	Q_{10}
	Range (hrs.)	Mp_{30} (hrs.)	Range (hrs.)	Mp_{20} (hrs.)		
1	15–17	16	24–26	25	9	1.56
2	23–24	23.5	36–48	42	18.5	1.79
3	24–26	25	46–50	48	23	1.92
4	31–33	32	62–64	63	31	1.97
5	23–24	23.5	46–48	47	23.5	2.00
6	23–25	24	47–49	48	24	2.00
7	23–24	23.5	47–49	48	24.5	2.04
8	51–53	52	108–120	114	62	2.19
9	18–20	19	41–43	42	23	2.21
10	17–19	18	40–42	41	23	2.28
11	27–31	29	65–72	68.5	39.5	2.36
12	23–24	23.5	55–57	56	32.5	2.38
13	22–24	23	52–62	57	34	2.48
14	14–23	18.5	48–50	49	30.5	2.65
15	15–17	16	42–44	43	27	2.69
16	25–27	26	70–72	71	45	2.73
17	25–27	26	73–75	74	48	2.85
18	15–17	16	46–48	47	31	2.94
19	13–15	14	43–45	44	30	3.14
20	25–29	27	87–91	89	62	3.30
21	25–29	27	98–109	103.5	76.5	3.83
22	11–13	12	46–50	48	36	4.00
23	13–14	13.5	55–57	56	42.5	4.15
Mean	22.96		57.57		*34.61	2.59
S.E.	±1.73 hrs.		±4.39 hrs.		±3.31 hrs.	±0.14
S.D.	8.10 hrs.		20.59 hrs.		15.34 hrs.	0.67

* $t = 10.46$; $P = <.0001$.

transferred to the lower temperature. The results were in all respects similar to those of the preceding experiments, a much longer time being required by each fish for the disappearance of the band at the lower temperature. From a consideration of the Q_{10} values it can be seen that in the majority of cases the rate of the reaction was more than twice as high at the 30-degree level as at 20°, the mean ratio being 2.59 ± 0.14.

Discussion

In *Fundulus heteroclitus*, the time required for the complete blanching of caudal bands of equal width varies greatly among different individuals, even when maintained under identical environmental conditions during the process. This variation was apparent in the preliminary studies of this investigation and was confirmed by all subsequent work. On the other hand, each fish manifests a characteristic reaction time for the process. Thus, two bands of equal width produced upon the same individual were found to fade at remarkably similar rates under the same environmental conditions, the difference in time required for their disappearance being of no statistical significance.

This characteristic reaction time for individuals makes it possible, by performing "paired" experiments upon each of a number of animals, to determine with considerable accuracy the relation of a particular variable factor to the time required for the fading of caudal bands in Fundulus. In this way one band of each animal serves in effect as a control for the other and any significant difference between the blanching time of the two can be reasonably attributed to the factor which has been varied. This method was employed in the present investigation to determine the effect of temperature upon the rate of the fading reaction.

The rate of fading of caudal bands in Fundulus was found to be influenced directly by temperature, a rise of 10° over the intervals 15 — 25° C. or 20 — 30° C. resulting in an average increase of 2 to 3 times in the speed of the reaction. The results obtained in this investigation thus agree in this respect with those of Dalton and Goodrich (1937) on the paradise fish, *Macropodus opercularis*. These authors ascribed their results to the effects of temperature upon the rate of diffusion of the concentrating neurohumor into the band from adjacent regions of the tail. This interpretation would admittedly add considerable significance to the immediate value of results of this sort in the light of the neurohumoral hypothesis. Unfortunately, however, such a conclusion is difficult to reconcile with the prevalent concept that the effect of temperature changes upon the rate of diffusion processes is ordinarily of considerably less magnitude than the effect observed upon the rate of disappearance of caudal bands in their study and in the present investigation. Jacobs (1928), for instance, states that ". . . processes whose rate is limited by the diffusion of dissolved substances (give) values of Q_{10} in the vicinity of 1.25 or 1.3; and chemical reactions of the ordinary sort values which are usually in excess of 2."

Hence, there is no *a priori* reason to regard the blanching reaction as one limited by diffusion, since its temperature coefficient falls within the range generally attributed to chemical reactions. This is not meant to imply, however, that diffusion is to be excluded from the total reaction. The blanching of caudal bands is undoubtedly a complex phenomenon, involving many different processes, several or possibly all of which may be affected by temperature. Consequently, the observed results may well represent the net effect of temperature upon the rate of the reaction as a whole rather than its influence upon any single process involved. It therefore seems advisable to regard the temperature coefficient of this reaction, like that of many other complex biological reactions, as being primarily of descriptive rather than of analytical value.

Summary

1. In *Fundulus heteroclitus*, the time required for the blanching of caudal bands of equal width varies greatly among different individuals under the same environmental conditions.

2. Each fish manifests a characteristic reaction time, however, and it is therefore possible, by performing "paired" experiments, to determine the effect of certain variable factors upon the rate of the blanching reaction in any individual.

3. By this method it has been found that the rate of blanching of caudal bands in Fundulus is directly influenced by temperature. A rise of 10° over the interval of 15° to 25° C. increased the speed of the reaction by an average of 3.04 ± 0.285 times in a group of 17 fish with one-ray bands. A similar rise over the interval of 20° to 30° C. increased it by an average of 2.18 ± 0.166 times in a group of 14 animals with one-ray bands, and 2.09 ± 0.16 times in seven animals with two-ray bands. Conversely, a decrease from 30° to 20° C. decreased the rate by an average of 2.59 ± 0.14 times in a group of 23 fish with one-ray bands.

It is suggested that these results be ascribed to the net effect of temperature upon the rate of the blanching reaction as a whole rather than to its influence upon any single process involved.

4. By the same method it was also found that the rate of blanching of caudal bands in fresh water is the same as that required in sea water. For a group of 8 animals with one-ray bands, the mean difference in reaction time in these two media at 30° C. was 0.44 ± 0.63 hours.

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THE CHROMATIN IN THE LIVING ARBACIA PUNCTULATA EGG, AND THE CYTOPLASM OF THE CENTRIFUGED EGG AS PHOTOGRAPHED BY ULTRA-VIOLET LIGHT

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Chromatin

In the living egg of *Arbacia punctulata*, as observed with high magnification with the best optical equipment for visible light, the mature nucleus in the unfertilized and recently fertilized egg appears clear and homogeneous. This is true in the normal egg where the red pigment somewhat obscures the picture, in the centrifuged whole egg where the pigment has been thrown down and the nucleus lies in the clear layer, and also in the white "half" (containing no pigment) obtained by centrifugal force. Nor can the chromosomes of the mitotic figure be seen in the living egg of Arbacia though the asters are very conspicuous. In sections of eggs, however, which have been fixed in Bouin's fluid and stained with Heidenhain's haematoxylin, the chromatin material is darkly stained and shows a characteristic structure, a network in the nucleus of the unfertilized and recently fertilized egg, and discreet bodies, chromosomes, during the mitotic divisions (Harvey, 1940).

A study of the chromatin material in the living egg of Arbacia as it appears with ultra-violet light has been made by the use of the quartz microscope devised by Köhler (1904), and modified by Lavin (1943). This is a microscope with quartz oculars and objectives so arranged that an accurate focus can be obtained on a fluorescent plate. The light source is a quartz spiral mercury resonance lamp (Hanovia Chemical Co.) from which the visible light is taken out by a liquid filter, so that only light of the wave length of 2537 A° is transmitted. The eggs were mounted on a quartz slide and covered with a quartz coverslip and partially compressed to make the eggs thin enough for the light to penetrate. Photographs of a selected field were taken with an exposure of three minutes. That the eggs were still living and coagulation had not set in was shown by progressive changes in serial photographs taken at intervals, of the same field of fertilized eggs.

We have arranged on Plate I photographs of similar stages of the egg of Arbacia: (1) Living eggs taken with ordinary visible light (Photographs 1, 4, 7). (2) Sections of eggs fixed in Bouin and stained with Heidenhain's haematoxylin, photographed with visible light (Photographs 2, 5, 8). (3) Living eggs photographed with ultra-violet light (Photographs 3, 6, 9). Three stages are shown, one of the immature egg with large germinal vesicle and nucleolus, a second of the mature egg with small nucleus, and a third of a late prophase or early metaphase. It will be seen that the chromatin material which does not show at all with visible light in the living egg is quite plain with the ultra-violet light and similar in appearance to the stained preparations with visible light.

The dark areas in the prints are, of course, the areas where the ultra-violet light is absorbed. The absorption is probably due to the presence of nucleic acid compounds since the purine and pyrimidine constituents of nucleic acid have an absorption maximum in this region of the spectrum. Of course this does not rule out the absorption due to the possible presence of proteins, but since the absorption of nucleic acid is much greater than that of the proteins, and until it is possible to differentiate between the two, the absorbing material will be referred to as nucleic acid compounds. It is not possible from the photographs to tell whether the absorbing materials are nucleic acids of the desoxyribose type (thymonucleic) or of the ribose type (yeast nucleic). The absorbing material in the immature egg (Photograph 3) is the nucleolus and a coarse network throughout the germinal vesicle; the bulk of the material in the germinal vesicle is non-absorbing, much less absorbing than the cytoplasm. In the mature nucleus (Photograph 6) the absorbing material is the chromatin network, and the rest of the material is less absorbing than the cytoplasm. In the dividing cell (Photograph 9) the chromosomes absorb and there is a considerable amount of non-absorbing material around them. There is no evidence of spindle or asters, such as one sees with visible light (Photographs 7, 8). There is the bare possibility that this may be due to the cell being somewhat compressed in order to allow the light to penetrate. The nucleic acid compounds of the nucleus, therefore, seem to be restricted to the nucleolus and network of the germinal vesicle of the immature egg, the chromatin threads of the mature nucleus and the chromosomes of the dividing egg.

It is generally agreed that the nucleic acid of the nucleus is of the desoxyribose type (thymonucleic) and that of the cytoplasm of the ribose type (yeast nucleic acid). The nucleolus is believed to be of the ribose type (Caspersson and Schultz,

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PLATE I

Photomicrographs, with approximate magnifications, as indicated, to bring comparative photographs to about the same size. Allowance is thus made for shrinking in fixation and expansion due to pressure of the coverslip for the ultra-violet.

PHOTOGRAPH 1. Living immature egg with visible light. 500 ×.

PHOTOGRAPH 2. Section of immature egg, fixed in Bouin and stained with Heidenhain's haematoxylin. Visible light. 660 ×.

PHOTOGRAPH 3. Living immature egg with ultra-violet light. 500 ×.

PHOTOGRAPH 4. Living mature unfertilized egg with visible light. 500 ×.

PHOTOGRAPH 5. Section of mature unfertilized egg, fixed in Bouin and stained with Heidenhain's haematoxylin. Visible light. 660 ×.

PHOTOGRAPH 6. Living mature unfertilized egg with ultra-violet light. 500 ×.

PHOTOGRAPH 7. Living fertilized egg at metaphase. Visible light. 500 ×.

PHOTOGRAPH 8. Section of fertilized egg at metaphase (early), fixed in Bouin and stained with Heidenhain's haematoxylin. 660 ×.

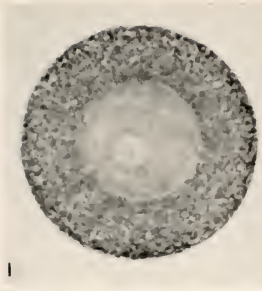
PHOTOGRAPH 9. Living fertilized egg at metaphase (early) with ultra-violet light. Egg somewhat squashed. 500 ×.

PHOTOGRAPH 10. Living egg centrifuged, 10,000 × g. for two minutes. Oil cap, clear layer, mitochondrial band, yolk, pigment. Nucleus under the oil cap. Visible light. 280 ×.

PHOTOGRAPH 11. Section of centrifuged egg, fixed in formalin and unstained. Visible light. 400 ×.

PHOTOGRAPH 12. Section of centrifuged eggs, fixed in formalin and stained with Heidenhain's haematoxylin. Visible light. 400 ×.

PHOTOGRAPH 13. Section of centrifuged eggs, fixed in formalin, and unstained. Ultra-violet light. 400 ×.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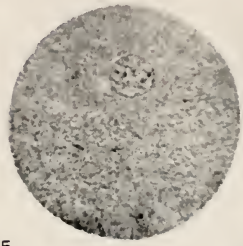
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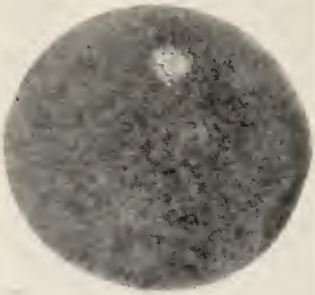
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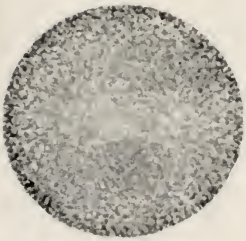
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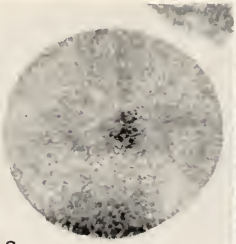
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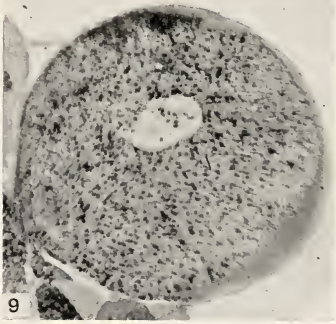
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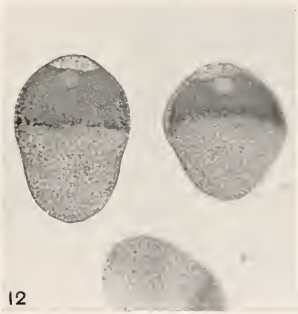
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PLATE I

1940). The spindle of the dividing cell probably does not contain nucleic acids of either type, though there is some disagreement about this (Stedman and Stedman, 1943a, b, c vs. Callan, 1943; Barber and Callan, 1944).

Blanchard (1935) extracted from masses of unfertilized mature Arbacia eggs nucleic acid of the desoxyribose type and also a pentose derivative whose properties resembled those of a ribose nucleic acid, both in approximately the same amount. Brachet (1933, 1937) found little of the desoxyribose type in unfertilized eggs of another sea urchin, *Paracentrotus lividus,* and thought that Blanchard's results were due to the presence of ovarian tissue, other than the eggs themselves, rich in the desoxyribose type. Brachet found that the desoxyribose nucleic acid increased in amount and the ribose type decreased after fertilization. According to Caspersson and Schultz (1940), there is an accumulation of ribonucleic acid in the cytoplasm close to the nuclear membrane in the ovarian (immature) egg of *Psammechinus miliaris,* another sea urchin; this they think indicates a "synthesis of nuclear products influencing cytoplasmic activity." In our ultra-violet photographs of Arbacia, one cannot distinguish between the ribose and desoxyribose nucleic acids since they absorb equally. In some of the photographs there is a distinct massing of strongly absorbing material, either as a clump or as a thick ring, on the outside of the nuclear membrane, but it is not constant.

The first work on photography with ultra-violet light on living and fixed (unstained) cells was done by Köhler in 1904 with his original ultra-violet microscope. His photographs show very nicely the nuclei in living cartilage cells of Triton and the spireme threads and chromosomes of fixed epithelial cells of Salamandra. Very good ultraviolet photographs of grasshopper (*Melanoplus femur rubrum*) spermatocyte cells, showing spiremes and chromosomes in all stages of mitosis, were published by Lucas and Stark in 1931; they show no spindle fibers, though they state that these can be seen in unstained sections. Wyckoff and Ebeling (1933) show similar ultra-violet photographs of "grasshopper" spermatocytes. Caspersson (1936) shows similar photographs of such cells in other species of Orthoptera (*Chorthippus dorsatus* and *Gomphocerus maculatus*). These chromatic structures in Orthopteran sperm cells, however, show well in the living material with visible light (Chambers, 1914, in *Disosteira carolina;* Bělař, 1929, in *Chorthippus lincatus*). It is of interest that the giant chromosomes of the salivary gland cells of Drosophila show their banded structure well with ultra-violet light (Caspersson, 1936). In *Arbacia punctulata,* the chromatin network and chromosomes cannot be seen with visible light in the living egg, but show very nicely with ultra-violet light and appear exactly as they do in fixed and stained preparations. Judging from the absorption of ultra-violet light, compounds which absorb 2537 A° strongly are present in the Arbacia egg in the nucleolus, the chromatin network and the chromosomes, but not in the spindle fibers and asters, and not in the nuclear sap.

Cytoplasm of the Centrifuged Egg

When the *Arbacia punctulata* egg is centrifuged, the stratification is as follows: Oil at the centripetal pole, clear layer, a band of mitochondria, a large layer of yolk granules, and red pigment granules at the centrifugal pole; the nucleus lies in the clear layer under the oil cap (Harvey, 1932; 1940). In the living egg, the stratification is very striking (Photograph 10). The different materials stain selectively

with many vital dyes; e.g. the mitochondria stain purple with methyl green, the yolk and pigment stain blue with methylene blue (Harvey, 1941). When the centrifuged eggs are fixed, sectioned and stained with Heidenhain's haematoxylin, the layers are not so striking. After a Bouin fixation, the oil cap is not to be seen; it has apparently been dissolved by the fixative (Harvey, 1940, Photograph 124). However, with a formalin fixation the oil cap is preserved (Photographs 11, 12). The most striking difference between the living egg and the fixed and stained sections is in the clear layer. This is optically empty in the living egg, but is deeply stained in the haematoxylin preparations and is filled with very small granules (Photograph 12). In the formalin fixed sections, stained with haematoxylin, the different layers of mitochondria, yolk and pigment are scarcely distinguishable; they are apparent, however, in Bouin-fixed sections, especially when the haematoxylin preparations are counterstained (Harvey, 1940, Photograph 124).

It was found difficult to obtain good ultra-violet photographs of the living centrifuged egg, as it tended to burst with the pressure of the coverslip necessary to obtain the desired thinness for the light to penetrate. We therefore used unstained sectioned material; the Bouin fixative was not suitable for the ultra-violet, but the formalin fixation was quite satisfactory. The most absorbing area is the clear layer, which appears quite dark in the ultra-violet photographs, much darker than the nucleus (Photograph 13). A control, unstained, section photographed by visible light is shown in Photograph 11. The oil cap and the granular layers, mitochondria, yolk and pigment are somewhat but not markedly absorbing with ultra-violet light, and all about equally so. There is no special absorption by the mitochondria. The bulk of the protoplasmic nucleic acid compounds is therefore in the clear layer. This material is the ground substance or matrix of the uncentrifuged egg in which the granules lie. This is apparently also the important material for development, rather than the granules, since it has been shown that fractions of eggs, obtained by centrifugal force, may lack any one of the different types of granules and still develop (Harvey, 1932, 1936, 1940). It is of interest to find that it is the ground substance or matrix which contains the compounds which absorb in the nucleic acid region of the spectrum.

Summary

1. Photographs of living immature, mature and dividing eggs of *Arbacia punctulata,* taken with ultra-violet light of wave length 2537 A°, show an absorption, indicating presence of nucleic acid compounds, in the nucleolus, chromatin network and chromosomes. The photographs with ultra-violet light are similar to those of sections of fixed material stained with Heidenhain's haematoxylin taken with visible light. The chromatin network and chromosomes cannot be seen with visible light in the living egg.

2. In the centrifuged egg, with ultra-violet light, the clear layer shows greatest absorption, indicating the localization of nucleic acid compounds in this layer. With visible light, this layer appears dark and granular in sections stained with Heidenhain's haematoxylin, much as it does with ultra-violet light. It is optically empty in the living centrifuged egg with visible light. This layer represents the matrix or ground substance of the normal uncentrifuged egg. The matrix of the nucleus and the layers of granules (mitochondria, yolk, pigment and oil) are relatively non-absorbent with ultra-violet light.

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XANTHOPHYLLS AND CAROTENES OF DIATOMS, BROWN ALGAE, DINOFLAGELLATES, AND SEA-ANEMONES

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Diatoms, dinoflagellates and brown algae, the principal autotrophic organisms of the sea, contain chlorophyll *a*, chlorophyll *c* (Strain and Manning, 1942a; Strain et al, 1943) and complex mixtures of yellow pigments. These yellow carotenoid pigments, which appear to play a role in photosynthesis (Dutton and Manning, 1941), have now been investigated by means of the highly selective chromatographic adsorption method. They have been compared with similar xanthophylls and carotenes from other sources such as leaves, flowers, and sea-anemones.

In the past seventy-five years there have been many investigations of the carotenoid pigments of diatoms and of brown algae. β-Carotene is the principal carotene and fucoxanthin is the principal xanthophyll (Palmer, 1922; Kylin, 1927; Walker, 1935; Heilbron, 1942). One or two additional fucoxanthin-like xanthophylls (Kylin, 1927; Montfort, 1940; Seybold and Egle, 1938; Seybold et al, 1941; Handke, 1941) have been regarded as native pigments (Kylin, 1927), as oxidation products of the principal fucoxanthin (Heilbron and Phipers, 1935; Kylin, 1939) or as interconvertible fucoxanthin isomers (Strain and Manning, 1942b). Other xanthophylls reported in brown algae are: fucoxanthophyll (Tswett, 1906a), xanthophyll or lutein (Palmer, 1922; Kylin, 1927; Heilbron, 1942; Montfort, 1940; Seybold and Egle, 1938; Seybold et al, 1941; Handke, 1941; Heilbron and Phipers, 1935; Kylin, 1939; Willstätter and Page, 1914; Carter et al, 1939; Pace, 1941), phyllorhodin or zeaxanthin (Kylin, 1939), cryptoxanthin and isolutein (Pace, 1941). Phylloxanthin, first reported both in brown algae and in leaves of higher plants, was found to be similar to the violaxanthin of pansy flowers (Kylin, 1927; 1939; Heilbron, Parry and Phipers, 1935). Zeaxanthin has also been regarded as a post-mortem product of brown algae (Heilbron and Phipers, 1935). Additional xanthophylls of diatoms have been reported as xanthophyll or lutein (Carter et al, 1939; Palmer, 1922; Kylin, 1927; Heilbron, 1942; Montfort, 1940; Seybold and Egle, 1938; Seybold et al, 1941; Handke, 1941; Pace, 1941), zeaxanthin, cryptoxanthin and isolutein (Pace, 1941).

There have been few investigations of the xanthophylls of dinoflagellates. The principal xanthophyll, called *peridinin* (Schütt, 1890; Kylin, 1927; Seybold et al, 1941), resembles the pigment sulcatoxanthin subsequently isolated from sea-anemones (Heilbron, Jackson and Jones, 1935). Another pigment of dinoflagellates is similar to the strongly adsorbed xanthophylls prepared from higher plants (Kylin, 1927; Strain, 1938a). The presence of lutein (xanthophyll) has also been reported (Seybold et al, 1941).

Experimental

Plant material. Brown algae were collected on the rocky reefs near Half Moon Bay, California. They were: *Fucus furcatus, Hesperophycus Harveyanus, Pelvetiopsis limitata, Cystoseira Osmundacea, Laminaria Andersonii, Pterygophora californica, Egregia Menziesii, Macrocystis integrifolia, Nereocystis pyrifera.*

Two colonial diatoms collected in quantity at the seashore were *Navicula torquatum* and *Isthmia nervosa.* Other diatoms, grown in pure culture or in unialgal culture, included *Nitzschia closterium, Nitzschia palea, Stephanopyxis turris* and *Thalassiosira gravida* (Strain and Manning, 1942a; Strain et al, 1943).

A fresh-water dinoflagellate, *Peridinium cinctum* (Strain et al, 1943), and a fresh-water yellow-green alga, *Tribonema bombycinum,* were found growing in a high state of purity as natural "blooms." A small, unicellular, brown colored alga was obtained in great concentration from the tentacles of the large, common, Pacific Coast sea-anemone *Bunodactis* (*Cribrina*) *xanthogrammica* (Strain et al, 1943). The amounts of algal material employed for preparation of the xanthophylls varied from 1 or 2 gm. of the centrifuged unicellular algae to 15 or 20 gm. of the brown algae. Only fresh, living algae were utilized.

Extraction of pigments. Absolute methanol containing about 0.5 per cent dimethylaniline was usually employed to extract the pigments from algae (Strain, 1938b; Strain and Manning, 1942a; Strain et al, 1943). Alteration of the chlorophyll to products that were difficult to remove from the xanthophylls was retarded by extraction of the plant material at room temperature with such a large quantity of methanol that the total amount of water present was not over 2 to 5 per cent.

Chromatographic behavior of pigments. Separation of the xanthophylls from the chlorophylls in extracts of many algae through use of saponification methods was not feasible, because some of the xanthophylls were altered by alkali. Partition of the pigments between methanol and petroleum ether failed to remove alcohol-soluble chlorophyll *c* from the xanthophylls. Modifications of the chromatographic adsorption method were finally utilized for separation of the chlorophylls and carotenes from the xanthophylls and for further resolution of the xanthophylls and carotenes.

No single adsorption procedure sufficed to separate all the algal pigments from one another. Many of the pigments were decomposed slowly by adsorption upon magnesium oxide. After partial separation of the pigments by adsorption upon columns of sugar, the incompletely separated, weakly adsorbed xanthophylls could be resolved further by adsorption upon columns of magnesia or by readsorption upon columns of sugar using mixtures of various solvents for development of the chromatograms. Under the different conditions, the relative positions occupied by the xanthophylls on the columns were often quite different (Strain, 1942a; 1942b; LeRosen, 1942).

Chromatographic preparation of pigments. Pigments contained in the methanol extracts of algae were transferred to petroleum ether by the addition of this solvent and aqueous salt solution. The green petroleum ether solutions were washed with water to remove residual methanol, concentrated to about 40 ml. at a temperature never above 20°, and then filtered through adsorption columns of dry powdered sugar (Strain, 1942a; 1942b; Strain et al, 1943), usually 3 or 4 cm. by 20 to 27 cm. In order to carry the last portions of the weakly adsorbed carotenes

below the other pigments, the columns were then washed with a little fresh petroleum ether, followed by petroleum ether containing 0.5 per cent *n*-propanol and 0.5 per cent dimethylaniline.

For spectroscopic examination of the carotene mixture small portions of the percolates were evaporated to dryness at reduced pressure. The residual carotenes were dissolved in 95 per cent ethanol, and the absorption spectra of the solutions were determined. For further identification of the carotenes, the bulk of the petroleum ether percolates were extracted several times with very dilute hydrochloric acid (0.5 per cent) which removed the dimethylaniline. The carotenes were then identified by adsorption upon a column prepared from a mixture of Micron Brand magnesium oxide No. 2641 and heat-treated diatomaceous earth (Filter Aid 501) (Strain, 1938a; 1942b).

Continued washing of the sugar columns with petroleum ether containing 0.5 per cent *n*-propanol caused the weakly adsorbed xanthophylls to separate from the chlorophyll *a* and from the strongly adsorbed xanthophylls and chlorophyll *c*. Resolution of the strongly adsorbed xanthophylls proceeded much more rapidly when petroleum ether with about 2 per cent *n*-propanol was subsequently used to develop the chromatogram. In order to accelerate the separation of the pigments and to avoid contamination of the resolved compounds with isomerization products that formed slowly, the weakly adsorbed and the strongly adsorbed xanthophylls were usually prepared rapidly on separate columns. For similar reasons, only freshly prepared extracts of the algae were utilized.

Petroleum ether solutions of several different alcohols were tested as solvents for the separation of algal pigments adsorbed upon columns of sugar. The resolving effect of aliphatic alcohols of various molecular weights was roughly equivalent to the amount of hydroxyl group that was present in the petroleum ether solution. Alcohols of higher molecular weight, such as amyl alcohol, produced slightly better separations of the pigments than alcohols of low molecular weight. At concentrations above 3 per cent, methanol tended to separate from the petroleum ether as a distinct phase in the adsorption columns. Alcohols of high molecular weight remained in solution, but they were difficult to remove from the petroleum ether by extraction with water and thus hindered further purification of the pigments by readsorption.

Pigments separated upon the adsorption columns were often contaminated by trailing portions of the substances which had preceded them (Strain, 1942a). All pigment preparations were purified by readsorption under various conditions until superposable spectral curves were obtained. The small quantities of the readsorbed pigments and the lability of many of them precluded the use of purification procedures involving crystallization.

Determination of properties of xanthophylls and carotenes. The most useful properties proved to be *partition between immiscible solvents* such as aqueous methanol and petroleum ether, *color reactions* produced by strong acids such as concentrated hydrochloric acid in the presence of ether, *comparative adsorbabilities* usually determined by adsorption upon Tswett columns of a mixture by the substances to be compared (Strain, 1942b), and *spectral absorption characteristics.*

Spectral absorption properties of the xanthophylls, determined with a photoelectric spectrophotometer (Smith, 1936), are plotted as the so-called characteristic absorption curves, the plot of log log (I_0/I) *versus* wave-length. Because of the

Color of adsorbed pigments	Name of pigments	Wave-lengths of absorption maxima (ethanol, mμ)	
Light green	Chlorophyll *c*		
Orange	Neofucoxanthin A	447	
Yellow-orange	Neofucoxanthin B	446	
Orange	Fucoxanthin	453	
Yellow	Diadinoxanthin	448	478
Yellow	Diatoxanthin	453	481
Green	Chlorophyll *a*		
Traces of yellow, green and gray	Xanthophylls Chlorophyll *a'* Pheophytin *a*		
Yellow	Carotenes		

FIGURE 1. Diatom pigments separated by adsorption upon a column of powdered sugar.

lability of the pigments, the small quantities obtained, and the presence of colorless concomitants, determination of the specific absorption coefficients was not feasible. Relative absorption values were always determined within a few hours after purification of the pigments by adsorption.

Results

Xanthophylls of diatoms. All the diatoms yielded the same series of xanthophyll pigments shown in Figure 1. However, different species showed significant variations in the relative amounts of the pigments.

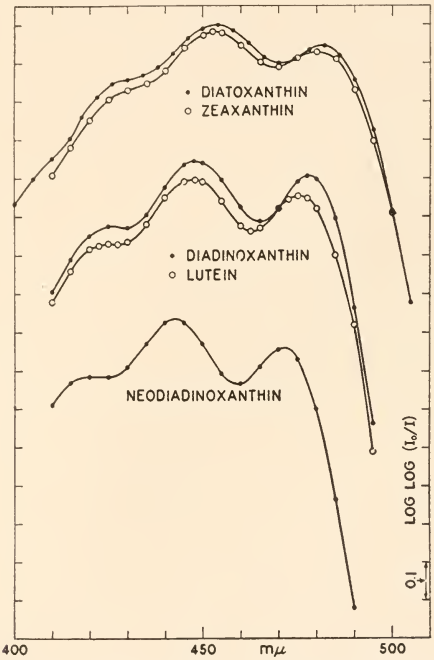


Figure 2. Characteristic spectral absorption curves of diatoxanthin, zeaxanthin, diadinoxanthin, luetin and neodiadinoxanthin. Solvent ethanol (95 per cent).

Diatoxanthin, a new xanthophyll, formed a paler but distinctly more orange band than the pigment adsorbed above it. Purified by readsorption, diatoxanthin yielded the spectral absorption curve shown with the similar zeaxanthin curve (Strain, 1938a) in Figure 2.

When dissolved in ether and treated with concentrated hydrochloric acid, diatoxanthin did not yield colored products. A mixture of diatoxanthin and zeaxan-

thin (the latter from the calyx of *Physalis alkekengi*) (Strain, 1938a) adsorbed from petroleum ether on a column of sugar and washed with petroleum ether containing one per cent methanol was resolved very slowly, zeaxanthin forming the lower band.

Diatoxanthin, dissolved in *n*-propanol and heated on a boiling water bath for one hour, underwent reversible isomerization, yielding some *neodiatoxanthin*. This second pigment was more strongly adsorbed and formed a yellower band, on a column of sugar, than the unchanged diatoxanthin (petroleum ether with 0.5 per cent *n*-propanol as wash liquid). Spectral absorption maxima of neodiatoxanthin were less pronounced and occurred at wave-lengths about 6 mμ shorter than those of diatoxanthin. By analogy with isomerization reactions of other carotenoid pigments (Strain, 1941; Polgár and Zechmeister, 1942), neodiatoxanthin probably represents a labile modification of the polyene system present in the molecule, diatoxanthin representing the stable or *trans* configuration.

In respect to color and position on the sugar columns, adsorbed neodiatoxanthin resembled the yellow band containing diadinoxanthin found next above the diatoxanthin when pigments of diatoms were adsorbed (see Fig. 1). However, diadinoxanthin and neodiatoxanthin did not yield identical spectral absorption curves. When a mixture of the two pigments was adsorbed on a column of sugar (petroleum ether plus 0.5 per cent *n*-propanol as wash liquid), neodiatoxanthin was adsorbed above diadinoxanthin.

Except for slight interconversion, neither diatoxanthin nor neodiatoxanthin was altered by a strong solution of potassium hydroxide in methanol. The behavior of these two pigments upon heating their solutions indicates that neither one is a labile isomer of zeaxanthin. Not more than traces of neodiatoxanthin or of zeaxanthin could have been present in the diatoms.

Diadinoxanthin (see Fig. 1) resembled lutein in respect to its spectral absorption (see Fig. 2) and color reactions. Readsorbed with lutein upon a column of sugar (petroleum ether with 0.5 per cent *n*-propanol as wash liquid), the diadinoxanthin moved through the column only about two-thirds as fast as the lutein.

As illustrated in Figure 1, the leading portion of the band of adsorbed diadinoxanthin became quite diffuse while the upper boundary of the band remained sharp and concentrated. This unique distribution of the pigment adsorbed on the column was observed after the diadinoxanthin had been purified in various ways. Spectroscopic properties of the pigment from the leading and trailing portions of the band were identical. Readsorption of each of these fractions upon fresh columns of sugar again yielded bands with diffuse leading portions.

In solution in hot *n*-propanol, diadinoxanthin underwent rapid, reversible isomerization yielding the similar, more adsorbed *neodiadinoxanthin*. Spectral absorption properties of the two pigments may be compared in Figure 2.

Fucoxanthin and its isomers (Strain and Manning, 1942b) contained in the extracts of diatoms moved slowly through the adsorption columns as indicated in Figure 1. Instead of the names suggested previously for the isomeric fucoxanthins (Kylin, 1927; 1939; Strain and Manning, 1942b), it is now proposed to call the principal isomer *fucoxanthin*, the other isomers *neofucoxanthin* A and *neofucoxanthin* B in conformity with the nomenclature of isomeric carotenoid pigments introduced by Zechmeister and his co-workers (Polgár and Zechmeister, 1942; and incl. refs.).

When the pigments were extracted quickly from diatoms and adsorbed on sugar from solution in petroleum ether containing 2 per cent *n*-propanol, the same mixture of pigments was always obtained. Adsorption of the principal fucoxanthin isomer under these same conditions yielded only traces of the other two compounds. This indicated that all three fucoxanthins may be normal constituents of the diatoms.

Interconversion of the three fucoxanthins proceeded rapidly in ethanol at 76° and slowly at 20°. Interconversion was accelerated by light and by substances upon which the pigments were strongly adsorbed, as for example by powdered glass or sugar when petroleum ether was used as solvent. This interconversion was also catalysed by iodine, but when bases such as pyridine and dimethylaniline were not added to neutralize traces of hydriodic acid that were formed (Strain, 1941), other pigments were produced. About 90 per cent of the equilibrium mixture, obtained by heating a solution of any of the isomers, was the stable fucoxanthin.

When dissolved in ether and treated with concentrated hydrochloric acid, each of the fucoxanthin isomers was converted into acid-soluble blue compounds. Treated with alcoholic potassium hydroxide the isomers formed pale yellow pigments that gave a blue color reaction with very dilute hydrochloric acid. The reaction with alkali proceeded in vacuum and in hydrogen as well as in air.

In order to obtain consistent spectral absorption curves for the isomeric fucoxanthins it was necessary to develop the chromatograms directly with petroleum ether containing 2 per cent *n*-propanol and 0.5 per cent dimethylaniline and to *work rapidly*. With these precautions, the same spectral curves were obtained whether the pigments were prepared from the diatoms or by interconversion from any of the other isomers (see Fig. 3). Noteworthy is the presence of only one definite spectral absorption maximum in the curve of each pigment. The spectral absorption curve of a petroleum ether solution of the principal fucoxanthin isomer, which is also shown in Figure 3, exhibits very distinct maxima, in sharp contrast to the curve for an ethanol solution. These spectral properties correspond to those of other carotenoid pigments that contain carbonyl groups in conjugation with the polyene structure in the molecule (Heilbron and Lythgoe, 1936).

These results on the xanthophylls of diatoms are contrary to the numerous reports (*loc. cit.*) that lutein is a constituent of these organisms. It is probable that diadinoxanthin may have been mistaken for lutein, and diatoxanthin may have been mistaken for zeaxanthin.

Carotenes of diatoms. Five of the six diatom species investigated contained principally β-carotene with only traces of other more adsorbed polyene hydrocarbons. One species, *Navicula torquatum*, contained the weakly adsorbed ε-carotene in addition to β-carotene (Strain and Manning, 1943a).

Effect of light of various spectral qualities on the formation of xanthophylls in diatoms. Pure cultures of *Nitzschia closterium* which were grown continuously in light from fluorescent "snow white" tubular lamps exhibited a constant ratio between the several xanthophyll pigments. When these cultures were placed in the red light from neon tubular lamps there was a gradual increase in the proportion of diadinoxanthin. After 20 days, when the culture had increased manyfold, the proportion of diadinoxanthin to other pigments was nearly twice as great as that in the cells grown in light from white fluorescent lamps. At this time the proportions between the other xanthophylls had changed very little.

These experiments demonstrated that the proportions of the carotenoid pigments of this photosynthetically active organism vary in response to changes in the environment. Whether this change results from variation in the rate of synthesis or degradation of the different pigments or whether it results from accelerated growth of a strain of the organism that contains different proportions of the xanthophylls is not yet known.

Xanthophylls of brown algae. Adsorption of the pigments of brown algae upon columns of sugar yielded a series of bands similar to those obtained from diatoms except that there was much less yellow pigment between the fucoxanthin and the chlorophyll *a*. Traces of two pigments, adsorbed as pale, narrow bands above

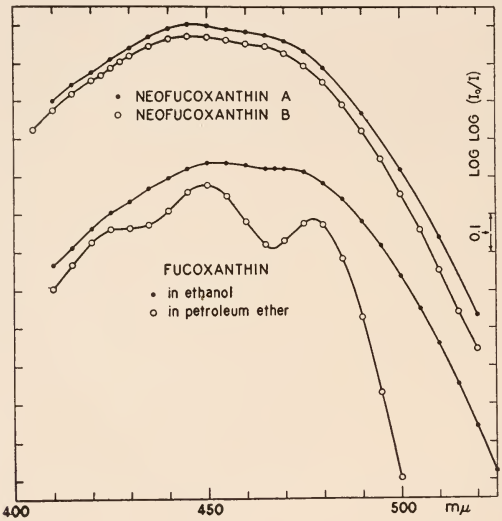


FIGURE 3. Characteristic spectral absorption curves of neofucoxanthin A, neofucoxanthin B and fucoxanthin dissolved in ethanol (95 per cent) and of fucoxanthin dissolved in petroleum ether.

chlorophyll *a*, appeared to be the diatoxanthin and the diadinoxanthin separated from the extracts of diatoms. The relative amounts of these two xanthophylls varied in different extracts of the same organism and in different species of brown algae. In view of the very small quantities of these two xanthophylls and their apparent absence in many species, it is uncertain whether the pigments came from the brown algae themselves or from diatoms that might have contaminated them.

When the pigments from brown algae were first adsorbed, a distinct yellow band usually appeared just below the orange fucoxanthin band. As the column was developed with petroleum ether containing propanol, this yellow band was often over-run by the orange fucoxanthin band. Under these conditions, the yellow pig-

ment, which exhibited spectral properties similar to those of violaxanthin, was observed again between the fucoxanthin and the neofucoxanthin B. If, however, the adsorbed pigments were washed with petroleum ether containing about 5 per cent acetone, the yellow band continued to advance ahead of the fucoxanthin, occasionally separating into two contiguous yellow bands. Pigment from the lower of these two yellow bands resembled the violaxanthin of leaves (Strain, 1938a), as is shown by the spectral absorption curves in Figure 4. Pigment from the upper yellow band exhibited a spectral absorption curve which (when corrected for absorption due to remaining violaxanthin) resembled the spectral curve of the flavoxanthin of leaves

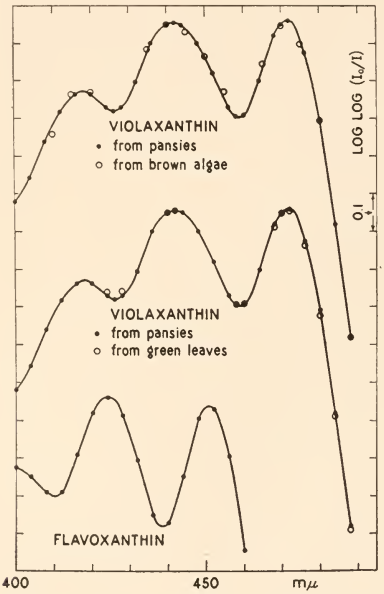


FIGURE 4. Characteristic spectral absorption curves of flavoxanthin and of violaxanthin from pansies, leaves and brown algae. Solvent, ethanol (95 per cent).

(Strain, 1938a). This flavoxanthin-like xanthophyll occurred in such small quantities that it could not be compared in other respects with the similar pigment of leaves.

The violaxanthin-like pigment from brown algae was found to be spectroscopically identical with violaxanthin prepared from leaves and from pansy flowers as described below (Fig. 4). Violaxanthin preparations from the several sources were also chromatographically identical. Mixtures of these preparations were inseparable by adsorption upon columns of magnesia (petroleum ether with 25 per cent acetone as wash liquid) or by adsorption upon columns of sugar (petroleum ether with 0.5 per cent *n*-propanol as wash liquid).

Changes in the relative positions of violaxanthin and of fucoxanthin, first observed when extracts of brown algae were adsorbed upon columns of sugar with different solvents as wash liquids, have been obtained with highly purified preparations of these two xanthophylls. With petroleum ether or with petroleum ether containing 5 per cent acetone as solvent, violaxanthin was adsorbed below fucoxanthin. With petroleum ether containing 0.5 per cent *n*-propanol as solvent, violaxanthin was adsorbed above fucoxanthin. Separated below fucoxanthin by adsorption from solution in petroleum ether containing acetone, violaxanthin gradually became adsorbed above the fucoxanthin when the column was washed with petroleum ether containing *n*-propanol.

Fucoxanthin and its isomers separated from the brown algae were identical with those separated from the diatoms as indicated by identical spectral absorption curves, adsorbability, and color reactions. Pigments that were extracted rapidly from fresh algal material and adsorbed from petroleum ether containing 2 per cent *n*-propanol yielded all three isomers; hence, these three pigments probably represent normal constituents of brown algae as well as of diatoms.

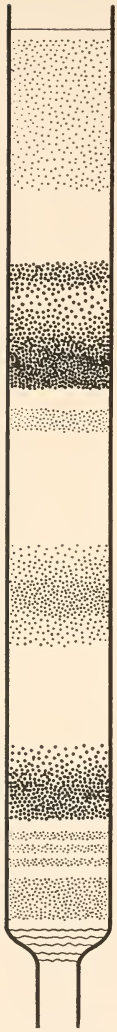
When the pigments of brown algae were adsorbed from solution in petroleum ether containing 2 per cent *n*-propanol, traces of xanthophylls with absorption spectra similar to that of neoxanthin from leaves (Strain, 1938a) were observed below and just above the fucoxanthin. Because of their weak adsorbability, neither of these pigments could have been neoxanthin. Their relations to other xanthophylls of similar spectra (Fig. 6) were not established. Traces of a xanthophyll with spectral properties similar to those of dinoxanthin (*vide infra*) were also observed on the column below the adsorbed fucoxanthin.

Carotenes separated from the brown algae were composed principally of the *beta* isomer with traces of more adsorbed carotenes. Neither α-carotene nor ϵ-carotene was observed.

These results on the carotenoid pigments of brown algae support the early observation of Tswett that fucoxanthophyll, the xanthophyll of these organisms, in addition to fucoxanthin, is different from xanthophyll (lutein) of leaves. They confirm the statement of Kylin (1927; 1939) that violaxanthin (phylloxanthin) is present in brown algae. They are in disagreement with the claims of Kylin and of many others, that lutein and zeaxanthin occur in many species of brown algae (some of which belong to the same genera as several of the species examined by us). In certain species of Laminaria and of Fucus, Heilbron and co-workers (Heilbron, 1942; Heilbron and Phipers, 1935; Carter et al, 1939) could not detect any xanthophylls other than fucoxanthin.

Our observations both on diatoms and on brown algae support Kylin's original statement (1927) and other recent claims (Montfort, 1940; Seybold and Egle, 1938; Strain and Manning, 1942b) that the several fucoxanthins are normal constituents of the plant cells. They are at variance with Heilbron and Phipers' contention that the additional fucoxanthin they observed is an oxidation product of the principal fucoxanthin. All these conclusions are difficult to reconcile with Kylin's latest statement (1939) that the pigment he regarded as fucoxanthin B may also have been an oxidation product. Zeaxanthin, reported as a normal constituent of brown algae by Kylin and by Pace and as a post-mortem product by Heilbron and Phipers, was not detected in our experiments.

Kylin (1939) has asserted that the violaxanthin of brown algae and of leaves



Color of adsorbed pigments	Name of pigments	Wave-lengths of absorption maxima (ethanol, mμ)	
Light green	Chlorophyll *c*		
Red-orange	Neoperidinin	464	
Red-orange	Peridinin	475	
Yellow	Neodinoxanthin	438	466
	Neodiadinoxanthin	442	471
Yellow	Dinoxanthin	441.5	471
	Diadinoxanthin	448	478
Green	Chlorophyll *a*		
Traces of yellow, green and gray	Xanthophylls Chlorophyll *a'* Pheophytin *a*		
Yellow	Carotenes		

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FIGURE 5. Pigments of *Peridinium cinctum* (and of an alga from a sea-anemone) separated by adsorption upon a column of powdered sugar.

is identical with Tswett's (1906b) β-xanthophyll, the only carotenoid observed above chlorophyll *b* when leaf pigments were adsorbed upon columns of precipitated chalk with carbon disulfide as wash liquid. Repetition of Tswett's experiment using extracts of sunflower leaves has revealed that neoxanthin (Strain, 1938a) with traces of other xanthophylls forms the topmost yellow band. On columns of sugar with petroleum ether containing 0.5 per cent *n*-propanol as wash liquid, neoxanthin also formed the topmost band. Only after prolonged development of the chromatogram did violaxanthin appear above the band of adsorbed chlorophyll *b*. Under these conditions traces of a flavoxanthin-like xanthophyll appeared between the neoxanthin and the violaxanthin. This indicates that Tswett's β-xanthophyll may have been a mixture containing principally neoxanthin.

Xanthophylls of a dinoflagellate. Pigments extracted from the dinoflagellate *Peridinium cinctum* and adsorbed on a column of sugar yielded the series of bands shown in Figure 5. The yellow band which occurred next above the chlorophyll *a* proved to be a mixture of at least two pigments which were separated from each other by readsorption upon a column of sugar (petroleum ether with 5 per cent acetone or 0.5 per cent *n*-propanol as wash liquid). Diadinoxanthin, which formed the lower band, was spectroscopically and chromatographically identical with this pigment prepared from diatoms.

Dinoxanthin, separated from the diadinoxanthin by readsorption of the mixture from the original column, exhibited spectral absorption properties similar to those of violaxanthin and of taraxanthin (Fig. 6). In spite of the similarity in their spectra, these three pigments are distinct substances as demonstrated by their different adsorbabilities and their color reactions with acid. When the pigments were adsorped on sugar from solution in petroleum ether containing one per cent *n*-propanol, the adsorption order was: violaxanthin (topmost), dinoxanthin, and taraxanthin. On magnesia with petroleum ether containing 25 per cent acetone as solvent, the adsorption order was: dinoxanthin, violaxanthin and taraxanthin. Dissolved in ether and treated with concentrated hydrochloric acid, dinoxanthin and taraxanthin yielded only a trace of blue color in the acid layer whereas violaxanthin yielded a stable, deep blue color in the acid. Prior treatment of dinoxanthin with alcoholic potassium hydroxide did not affect its reaction with concentrated hydrochloric acid. None of these pigments was interconvertible by the action of heat on its solution.

There were indications that traces of a flavoxanthin-like xanthophyll occurred in admixture with the diadinoxanthin and the dinoxanthin. The quantities present were insufficient to permit definite identification.

As illustrated in Figure 5, there was always a small yellow band just below the principal red-orange band obtained by adsorption of the pigments of the dinoflagellates. Readsorption of the pigment from this band on a fresh column yielded neodiadinoxanthin as the lower band and an isomer of dinoxanthin as the upper band. In Figure 6, a spectral absorption curve of this second pigment, *neodinoxanthin,* may be compared with the curve of the more stable interconvertible dinoxanthin. Color and solubility reactions of the two pigments were similar.

In all the experiments, even though rapid and mild conditions of extraction and adsorption were employed, both neodinoxanthin and neodiadinoxanthin were observed in the extracts of the dinoflagellates. Because of the ease and rapidity with

which these two isomers are formed, it is not certain that they are normal constituents of *Peridinium*.

Peridinin, obtained from the principal red-orange band (illustrated in Fig. 5), exhibited some noteworthy properties. So far as can be ascertained, this pigment

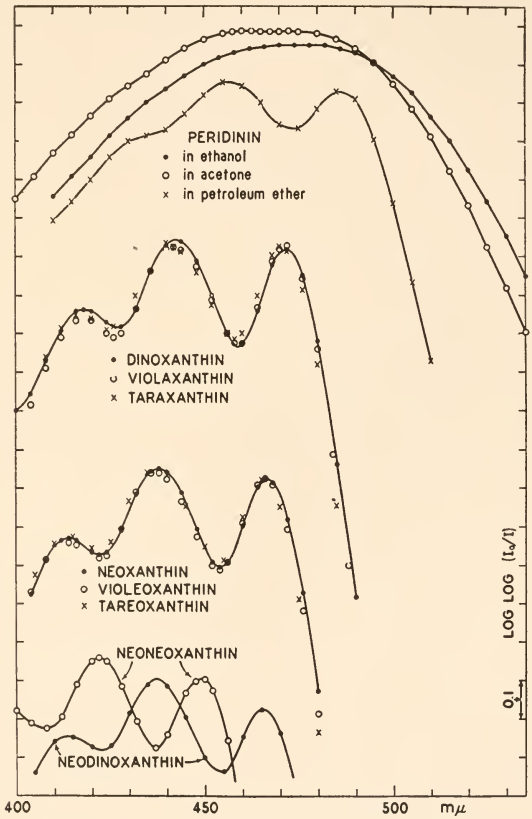

FIGURE 6. Characteristic spectral absorption curves of peridinin, dissolved in various solvents, and of dinoxanthin, violaxanthin, taraxanthin, neoxanthin, violeoxanthin, tareoxanthin, neodinoxanthin and neoneoxanthin dissolved in ethanol (95 per cent).

is the one observed in several species of *Peridinium* by Schütt (1890), by Kylin (1927), and by Seybold, Egle and Hülsbruch (1941). In solubility, peridinin resembled fucoxanthin, being extremely soluble in alcohols and but slightly soluble in petroleum ether. On sugar, it was much more adsorbed than fucoxanthin from

which it could be separated readily by development of the chromatogram with petroleum ether containing 2 or 3 per cent *n*-propanol. Peridinin was decomposed by alkalies, yielding paler pigments that did not give blue products when dissolved in ether and treated with concentrated hydrochloric acid. Peridinin itself, in contrast to fucoxanthin, did not yield a blue color with concentrated hydrochloric acid. This behavior of peridinin, like that of diadinoxanthin, is an exception to the rule that strongly adsorbed, alcohol-soluble xanthophylls yield a blue color with concentrated hydrochloric acid.

When dissolved in chloroform and treated with a solution of antimony trichloride in chloroform, peridinin formed a purple-orange solution that faded slowly to a lighter orange. Dissolved in chloroform and treated with concentrated sulfuric acid, peridinin caused the acid layer to turn deep blue. Crystals of peridinin, which formed readily when the solutions were concentrated, were turned deep blue by concentrated sulfuric acid.

Dissolved in methanol or in ethanol, peridinin exhibited strong absorption of light ranging from the violet to the yellow region of the spectrum. The spectral curve of alcohol and of acetone solutions showed a single broad absorption maximum. On the other hand, a solution of peridinin in petroleum ether exhibited two pronounced absorption maxima (see Fig. 6). Addition of as little as 2.5 per cent ethanol to a petroleum ether solution of peridinin caused a marked broadening of the absorption maxima. The petroleum ether solutions were quite yellow whereas the alcohol solutions were distinctly orange-red. By analogy with spectra of other carotenoid pigments (Heilbron and Lythgoe, 1936), these spectral properties indicate that the peridinin molecule contains at least one carbonyl group in conjugation with the polyene system.

Solutions of peridinin in carbon disulfide were pink-orange to red-orange in color. The peridinin in these solutions was very strongly adsorbed on sugar yielding a red adsorbate.

A dilute, yellow solution of peridinin in petroleum ether turned brick red when shaken with distilled water, and the pigment collected at the interface. This effect is similar to the color change observed when the peridinin is adsorbed on solids. Upon adsorption on solids or at a liquid-liquid interface, fucoxanthin also behaved similarly, but neoxanthin, which was more adsorbed than peridinin or fucoxanthin (see below), retained its characteristic yellow shade.

Peridinin, like other more typical xanthophylls, was moderately resistant to oxidation by atmospheric oxygen. Solutions of this xanthophyll in ethanol or in petroleum ether stored in open vessels in the dark were not bleached perceptibly in 2 or 3 months. In diffuse light from north windows, the solutions became colorless in a few weeks. In direct sunlight the solutions were decolorized in a few days.

When solutions of peridinin in *n*-propanol were heated on a boiling water bath, the pigment was isomerized rapidly. Products obtained by heating the solution for 2 hours were transferred to petroleum ether and adsorbed on a column of sugar using petroleum ether with 3 per cent *n*-propanol for development of the chromatogram. This resulted in the separation of a pale, diffuse red-orange band containing *neoperidinin* above the band containing the unchanged peridinin. Faint traces of other red-orange bands were adsorbed above the neoperidinin and very small quantities of still another pigment were adsorbed below the peridinin. In ethanol, all these pigments exhibited spectral absorption curves similar to that of peridinin but

their absorption maxima occurred at shorter wave-lengths. Traces of all these pigments were often observed when extracts of Peridinium were adsorbed upon columns of powdered sugar.

The carotene mixture extracted from *Peridinium cinctum* was composed almost entirely of β-carotene. The quantity of carotene present in the fresh centrifuged cells was greater than that obtained from the diatoms.

Xanthophylls of an alga from a sea-anemone. Algae squeezed from the tentacles of the sea-anemone, *Bunodactis xanthogrammica,* yielded the same series of pigments obtained from the dinoflagellates and tabulated in Figure 5. These pigments are constituents of the alga, not of the anemone.

Color reactions already described for peridinin from Peridinium were identical with those described for sulcatoxanthin from the sea-anemone *Anemonia sulcata* (Heilbron, Jackson and Jones, 1935). The absorption curve of peridinin dissolved in carbon disulfide exhibited two well-defined absorption maxima at 483 and 516 mμ, in good agreement with the values of 482 and 516 mμ reported for sulcatoxanthin. On the basis of available evidence, it appears that sulcatoxanthin is none other than the xanthophyll peridinin. As we have not been able to obtain the anemone *Anemonia sulcata* for analysis of its pigments, it is impossible to say whether the peridinin originates in the tissue of that animal or in the cells of the algae that are known to inhabit it (Fulton, 1922).

The identity of the pigments obtained from the symbiotic alga of Bunodactis and from the dinoflagellate suggests that both organisms may belong to the same or to related plant groups. From a microscopical examination of the symbiotic alga, Dr. H. W. Graham of Mills College concluded that this organism is not a dinoflagellate. It has been suggested that "zooxanthellae," the symbiotic algae of many sea anemones, may belong to another class of organisms, the cryptomonads (Fulton, 1922). If this is true, the autotrophic, free-living cryptomonads may also contain the same pigments found in the dinoflagellates.

Pigments of a yellow-green alga. The xanthophylls of *Tribonema bombycinum* proved to be different from those of all the other algae that we have investigated. Neither lutein nor any other common xanthophyll was observed. The several xanthophylls that were present formed pale yellow bands on the sugar columns, but these pigments have not been definitely identified. The carotene consisted almost entirely of the *beta* isomer. Tribonema did not contain chlorophylls *b, c,* or *d*.

Xanthophylls of dandelion flowers. For comparison with dinoxanthin, taraxanthin was prepared from flowers of the dandelion *Taraxacum officinalis* (Kuhn and Lederer, 1931). To this end, about 10 gms. of fresh dandelion flowers from which the stems and sepals had been cut were extracted with absolute ethanol; the extract was treated with an excess of potassium hydroxide; and after completion of the saponification, the xanthophylls were transferred to petroleum ether and adsorbed upon a column (3.7 by 22 cm.) of magnesia and siliceous earth (1:1). The chromatogram was then developed with petroleum ether containing 25 per cent acetone. Taraxanthin proved to be the principal, least adsorbed xanthophyll. After purification by readsorption it yielded the spectral absorption values shown in Figure 6.

Above the taraxanthin there were two or three adjoining bands one of which contained lutein. Above this group of bands, there appeared a yellow band which

yielded a hitherto undescribed pigment. This new xanthophyll, for which we propose the name *tareoxanthin,* exhibited a spectral curve almost identical in shape with that of neoxanthin from leaves (see Fig. 6) (Strain, 1938a).

Just above the band containing the tareoxanthin on the column with the adsorbed dandelion pigments there appeared a lemon yellow band. Xanthophyll eluted from this band exhibited a spectral absorption curve almost identical with that reported from flavoxanthin in ethanol (Strain, 1938a). Unlike flavoxanthin from other sources (Strain, 1938a; Kuhn and Brockmann, 1932), this xanthophyll from dandelions did not yield a blue product when dissolved in ether and treated with concentrated hydrochloric acid. This fact suggests that the "flavoxanthin" isolated from dandelion flowers by Karrer and Rutschmann (1942a) may not have been identical with flavoxanthin from other plants.

The mixture of dandelion xanthophylls was also resolvable by adsorption upon columns of sugar when petroleum ether containing one per cent *n*-propanol was used as solvent. Under these conditions the taraxanthin was adsorbed above the lutein.

Xanthophylls of pansy flowers. Violaxanthin, for comparison with the xanthophylls of algae, was prepared from the yellow flowers of *Viola tricolor* (Kuhn and Winterstein, 1931). Xanthophyll esters in the extracts of the flowers were saponified, and the free xanthophylls were crystallized from petroleum ether and aqueous alcohol. After recrystallization from petroleum ether and aqueous alcohol, the violaxanthin was purified further by adsorption on columns of magnesia and of sugar.

When the xanthophylls in the mother liquors from the violaxanthin preparation were adsorbed on magnesia or on sugar, relatively large quantities of a strongly adsorbed xanthophyll were observed (Karrer and Rutschmann, 1942b). This pigment, for which the name *violeoxanthin* is proposed, exhibited a spectral absorption curve almost identical with those of neoxanthin and of tareoxanthin (Fig. 6).

In spite of the similarity between the spectral absorption curves of neoxanthin, tareoxanthin, and violeoxanthin, these three pigments were readily separable by adsorption. Upon columns of magnesia with petroleum ether containing 25 per cent acetone as solvent or upon columns of sugar with petroleum ether containing one per cent *n*-propanol as solvent, neoxanthin formed the uppermost band, violeoxanthin the middle band, and tareoxanthin the lowest band. Dissolved in ether and treated with concentrated hydrochloric acid, violeoxanthin yielded an intensely blue, acid-soluble product whereas neoxanthin yielded no color, and the tareoxanthin yielded only traces of blue in the acid.

Violaxanthin from leaves. Violaxanthin was rapidly preparable from leaves by adsorption of a petroleum ether solution of the extracted pigments upon a column of magnesia and siliceous earth followed by development of the chromatogram with petroleum ether containing 25 per cent anhydrous acetone. Under these conditions, the violaxanthin was adsorbed far below the chlorophylls and below lutein. As a consequence it was not contaminated with other leaf pigments and was nearly spectroscopically homogeneous after a single adsorption.

Neoxanthin from leaves. When the pigments extracted from leaves were dissolved in petroleum ether and adsorbed upon a column of heat-treated siliceous earth (Filter Aid 501), the chlorophylls and xanthophylls were more adsorbed than upon a column of sugar. As the adsorbed pigments were washed with petroleum

ether containing 0.5 per cent *n*-propanol, neoxanthin formed the uppermost yellow band. Owing to the high filtration rate of the columns, considerable neoxanthin could be separated in a few minutes. Purified by readsorption upon fresh columns, the neoxanthin exhibited a spectral absorption curve identical with that of material prepared by adsorption upon magnesia from solution in dichloroethane (Strain, 1938a). It did not exhibit a blue color when dissolved in ethyl ether and treated with concentrated hydrochloric acid.

Dissolved in *n*-propanol and heated on a boiling water bath for several hours, neoxanthin was converted into two isomers that were more adsorbed than the unchanged neoxanthin. The most adsorbed isomer, *neoneoxanthin* A, exhibited spectral absorption maxima at wave-lengths almost identical with those reported for the absorption maxima of flavoxanthin, but its absorption maxima were not so pronounced (Fig. 6). These isomerization experiments indicate that neoxanthin contains the stable form of the chromophoric polyene group.

Both violaxanthin and neoxanthin were obtained from leaves that had been extracted at room temperature, from those that had been killed by boiling and from those that had been extracted with alcohol containing much dimethylaniline. These xanthophylls were also obtained when the leaf extracts were adsorbed directly on magnesia, on sugar or on siliceous earth or when they were adsorbed after treatment with alcoholic potassium hydroxide. They were not formed from lutein when this xanthophyll was exposed to the conditions utilized for the extraction and separation of the leaf pigments. These observations provide further indication that neoxanthin and violaxanthin are normal constituents of green leaves.

Relative adsorbabilities of xanthophylls. In addition to those reversals in the relative positions of adsorbed xanthophylls already reported, dinoxanthin was found to be adsorbed above diadinoxanthin in columns of sugar (solvents: petroleum ether containing 0.5 per cent *n*-propanol, 5 per cent acetone or 8 per cent methylisobutylketone). But, on magnesia with acetone or methylisobutylketone as solvents, dinoxanthin was adsorbed below diadinoxanthin.

A mixture of lutein and zeaxanthin was not readily separable on a column of sugar when petroleum ether with 5 per cent acetone was used as solvent. Adsorbed upon magnesia from solution in petroleum ether containing 25 per cent acetone, zeaxanthin was adsorbed far above lutein.

TABLE I

Relative positions of xanthophylls on columns of magnesia and of sugar with various solvents

	Magnesia	Magnesia	Sugar	Sugar
Solvent.....	Dichloro-ethane	Petroleum ether + 25% acetone	Petroleum ether + 25% acetone	Petroleum ether + 1% *n*-propanol
Xanthophylls...	Neoxanthin Violaxanthin Zeaxanthin Lutein	[Neoxanthin * Zeaxanthin] Lutein Violaxanthin	Neoxanthin Violaxanthin [Zeaxanthin * Lutein]	Neoxanthin Violaxanthin [Zeaxanthin * Lutein]

* Brackets indicate pigments that did not separate into bands.

Relative positions of the xanthophylls in the columns have been found to vary with changes in either the solvent or the adsorbent. This is illustrated by the results summarized in Table I.

Because knowledge of the relative positions of known xanthophylls in the adsorption columns aids in the preparation and identification of these pigments from various sources, the adsorption orders of many of the xanthophylls described in this paper are listed in Table II. For these comparisons, mixtures of two or three of

TABLE II

Relative positions of xanthophylls on columns of magnesia and of sugar

	Magnesia	Sugar
Solvent	Petroleum ether + 25% Acetone	Petroleum ether + 0.5–1% *n*-propanol
Xanthophylls	Neoneoxanthin A Neoneoxanthin B [Neoxanthin * [Zeaxanthin Fucoxanthin Peridinin [Violeoxanthin * [Lutein Dinoxanthin [Tareoxanthin * [Violaxanthin Taraxanthin Cryptoxanthin	Neoxanthin Peridinin Neofucoxanthin A Neofucoxanthin B Violeoxanthin Violaxanthin Fucoxanthin Tareoxanthin Taraxanthin [Zeaxanthin * [Lutein Cryptoxanthin

* Brackets indicate pigments that did not separate into discrete bands.

the xanthophylls, dissolved in petroleum ether, were adsorbed, and the chromatograms were then developed with petroleum ether containing the polar solvent.

Changes in the relative adsorbabilities of the xanthophylls with variations of the solvent are apparently related to the solubilities of the pigments as well as to effects of the solvents upon the chromophoric groups (see Fig. 6). For example, peridinin and fucoxanthin, which are adsorbed below zeaxanthin when acetone is used as solvent, are more soluble in this ketone than is zeaxanthin.

DISCUSSION

Algal pigments. A number of new xanthophylls have been isolated from the algae investigated. Additional pigments might be obtained by the use of larger quantities of plant material followed by readsorption of the minor bands on smaller columns. Utilization of additional refinements in technique may result in further resolution of the pigments described in this paper, especially the isomerization products of the stable xanthophylls. However, it seems improbable that such pigments would represent a considerable proportion of the total xanthophylls.

The relatively great adsorbability of the algal xanthophylls indicates that these hydroxy compounds do not occur in the form of esters. In this respect, xanthophylls of algae resemble those of the chloroplasts of higher plants (Strain, 1938a).

Occurrence of the unesterified xanthophylls in the photosynthetic apparatus contrasts with the occurrence of esterified xanthophylls in the yellow chromoplasts of fruits and flowers.

Among the algae, the xanthophylls have been found to vary in three ways. In a given species, the proportions of the several pigments may alter in response to changes in the environment. In different plant classes, the xanthophylls differ in kind as well as in quantity. In some plant groups spatially unstable xanthophylls occur along with the stable or *trans* xanthophylls.

Of all the pigments now considered to comprise a part of the photosynthetic apparatus of autotrophic plants, the xanthophylls vary most. Except for a few specialized, pigmented bacteria, autotrophic plants contain chlorophyll *a* and β-carotene as the principal representatives of these two ubiquitous types of plant pigments. By contrast, several groups, such as the diatoms, dinoflagellates, yellow-green algae and higher plants, do not contain a single xanthophyll in common (Strain and Manning, 1943b).

Because of the abundance and wide distribution of diatoms and dinoflagellates, the principal xanthophylls of these organisms, fucoxanthin and peridinin, must comprise a major portion of the total amount of xanthophylls occurring in the world's vegetation. In the dinoflagellate, the proportion of peridinin relative to the two chlorophylls was quite large. On account of the strong absorption of light by peridinin in the spectral region where absorption by the chlorophylls is weak, studies of the photosynthetic activity of dinoflagellates in light of various wavelengths might yield information regarding the role of this unique xanthophyll in photosynthesis.

Interpretation of the photochemical activity of the individual leaf pigments depends upon knowledge of the physical state and spectral properties of the several pigments in the leaf itself. Spectral properties of the pigments determined in any one solvent or in colloidal suspension may not yield results precisely applicable to calculation of the absorption by each pigment in the living organism.

The variation in pigments between members of different algal classes, together with the constancy of the pigments in different members of a single class, provides a promising approach to the problem of phylogenetic relationship between the various classes of algae. For example, brown algae contain at least one xanthophyll, violaxanthin, common to the higher green plants, whereas diatoms and dinoflagellates contain none. Tribonema, sometimes considered closely related to the diatoms, contains none of the principal diatom xanthophylls, nor chlorophyll *c*. None of the algae investigated contain lutein, the common xanthophyll of green algae and of higher plants. These and other phylogenetic aspects of our observations on the chloroplast pigments of plants will be discussed elsewhere.

Adsorption phenomena. Reversal of the relative positions of adsorbed substances with changes in solvents and adsorbents indicates disproportionate variations in the adsorbability. It follows that the relative adsorbability of chemical substances is not determined by chemical structure alone; hence, great care must be exercised if deductions of molecular structure are to be based upon relative adsorbability (Strain, 1942a; 1942b).

Variations of the relative adsorbabilities of chemical compounds with changes in adsorbents and solvents suggest precautions to be employed in the use of the chromatographic adsorption method. For instance, if one solvent causes a given

pair of substances to be adsorbed in one sequence and if another solvent causes the same substances to be adsorbed in the inverse order, then there should be at least one mixture of the two solvents that will not effect a separation of the pair of adsorbed substances. Tests of the selectivity of a given adsorbent for separation of a mixture of given compounds can not, therefore, be regarded as conclusive unless different solvents and various mixtures of these solvents are used to develop the chromatograms. Determination of the homogeneity of chemical substances and the comparison of substances suspected of being identical through use of chromatographic adsorption methods (Strain, 1942b) will be most effective when various adsorbents and solvents are employed. Reversal of relative adsorbability with changes in the solvents suggests that the selectivity of the adsorbent may be just as dependent upon the nature of the solvents employed as upon the inherent properties of the adsorbent itself. Unless special precautions are taken, these changes in relative adsorbability increase the chances for confusion and error when substances are identified by the so-called mixed chromatogram (Strain, 1942b).

Most of the plant pigments that have been separated upon adsorption columns have yielded bands or zones with the highest concentration of pigment in the leading portions and with diminishing concentrations in the trailing portions (Strain, 1942a). The inverse behavior of adsorbed diadinoxanthin is difficult to interpret in relation to the theories regarding the distribution of an adsorbed pigment upon the columns (Strain, 1942a; DeVault, 1943).

The great color change observed upon adsorption of fucoxanthin and peridinin, in contrast to the slight change observed upon adsorption of neoxanthin, may result from interaction between the adsorbent and the pigments rather than from increased concentration of the xanthophylls at the interface. This effect may be analogous to that produced by addition of polar solvents to solutions of peridinin in nonpolar solvents (Figs. 3 and 6). It emphasizes the desirability of additional knowledge concerning the state of the pigments within the leaf, especially for interpretation of the photosynthetic reactions in various spectral regions (Dutton and Manning, 1941) and for interpretation of color changes that occur when algae are killed. The strong adsorption of some of the xanthophylls at the interface between water and petroleum ether may be utilized for separation of these pigments from other less adsorped constituents in the extracts of plants (Strain, 1943).

Summary

Carotenoid pigments of several groups of algae have been obtained through utilization of the chromatographic adsorption method. The selectivity of this method and the relative positions of the pigments in the columns have been found to vary with the solvents and adsorbents that were employed.

Xanthophylls of algae represent a large proportion of the carotenoid pigments produced in the world's vegetation. Most of the algal xanthophylls were readily convertible, reversibly, into one or more isomers that were separated on the adsorption columns. The principal xanthophylls were the more stable, presumably *trans,* isomers. Some of the labile isomers also appeared to be normal constituents of the cells. All the algal xanthophylls were unesterified.

The following xanthophylls have been obtained from each of six species of diatoms: diatoxanthin, diadinoxanthin (both new xanthophylls), fucoxanthin, neo-

fucoxanthin A, and neofucoxanthin B. From some eight species of brown algae there were obtained: diatoxanthin (occasionally, in traces), diadinoxanthin (occasionally, in traces), violaxanthin, a flavoxanthin-like xanthophyll, fucoxanthin, neofucoxanthin A, neofucoxanthin B and traces of other xanthophylls. The dinoflagellate *Peridinium cinctum* and an alga inhabiting a sea-anemone yielded the following xanthophylls: diadinoxanthin, dinoxanthin (a new xanthophyll), neodiadinoxanthin (a new xanthophyll), neodinoxanthin (a new xanthophyll), peridinin, and two or three isomers of peridinin. A yellow-green alga, *Tribonema bombycinum*, did not contain fucoxanthin, peridinin or chlorophyll *c*.

None of the algae examined contained lutein. All the algae examined contained β-carotene as the principal polyene hydrocarbon. In only one species, the diatom *Navicula torquatum*, were appreciable quantities of another carotene ϵ-carotene, observed.

A new xanthophyll, tareoxanthin, was obtained from flowers of the dandelion. A flavoxanthin-like xanthophyll from these flowers may not be identical with flavoxanthin from other sources. Violeoxanthin, a new xanthophyll, was isolated from the flowers of the pansy. Improved methods for the isolation of neoxanthin and violaxanthin from leaves are described. The violaxanthin b of leaves and violaxanthin of pansies are chromatographically identical.

Several groups of xanthophylls with almost identical characteristic spectral absorption curves have now been found. Neoxanthin, tareoxanthin and violeoxanthin fall into one group; violaxanthin, taraxanthin, and dinoxanthin comprise another group; and there are indications of several flavoxanthin-like pigments.

The solvent has a pronounced effect on the shape of the spectral absorption curves of peridinin and of fucoxanthin. In addition to indicating an effect of the solvent on the structure of the pigment molecule, this phenomenon also emphasizes the importance of knowledge concerning the spectral properties of the xanthophylls in the living plant, especially if these spectral characteristics are to be employed in an analysis of the photochemical activity of the plant pigments.

Of the pigments comprising the photosynthetic apparatus, the xanthophylls are subject to the greatest variation. Nearly two dozen of these pigments have been found in the green parts of various plants.

All members of the Division of Plant Biology contributed invaluable discussion of the methods and results herein described. Dr. Gilbert M. Smith of Stanford University and Dr. H. W. Graham of Mills College aided in the identification of the algae.

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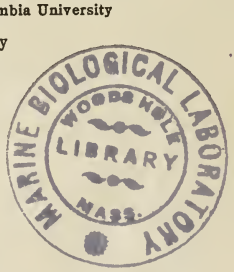

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